Biosensor based on directly immobilized hemoglobin and myoglobin

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Abstract: *Immobilization of proteins on a surface plasmon resonance (SPR) transducer is a delicate procedure since loss of protein bioactivity can occur upon contact with the untreated metal surface. Solution to the problem is the use of an immobilization matrix having a complex structure. In this paper we study the impact of direct immobilization of heme proteins (hemoglobin (Hb) and myoglobin (Mb)) on their bioactivity. We have used spin coating, for direct immobilization and matrix-assisted pulsed-laser evaporation (MAPLE) [1] for elaboration of the SPR biochip. The performance of both SPR chips – direct and MAPLE immobilized, was studied by SPR registration of the binding activity of Hb and Mb ligands with carbon monoxide (CO), carbon dioxide (CO2) and nitride oxide (NO). The experimental facts showed that direct immobilization of an intact molecule was achieved.*
KEYWORDS*: BIO-SENSING, MAPLE, PROTEIN*

1. Introduction

In addition to the great physiological importance of hemoglobin (Hb) and myoglobin (Mb), they play also an important role as effective recognition agents in chemical and biological sensors.
Hb is a protein with four polypeptide subunits and each subunit has an iron containing heme group. The activity of the porphyrin core determines the hemoglobin applications. Hb is widely used as a recognition medium in different biosensors. Variety of immobilization technics were developed and applied for NO_2 [2], NO_2 and H_2O_2 [3], $C_8H_8O_3$ [4], NO [5] and CO [6] sensing. All known sensing applications of Hb are realized as electrochemical sensors. Myoglobin (Mb) is a single-chain protein of amino acids containing a porphyrin complex with Fe in the center. The biological activity of Mb is based on the unique redox property due to the iron ion in its core. Thereby, Mb can be used to detect hydrogen peroxide (H_2O_2) [7], nitric oxide [8], hydrogen sulfide [9]. It is interesting that Mb has never been used for CO sensing although the prosthetic heme group can reversibly bind CO.

In this aspect the key factor in developing a reliable biosensor is the immobilization on the transducer. Usually immobilization can be produced by chemical methods, but there is frequently a need for homogeneous films of well-controlled thickness or films which can be deposited in a dry environment. The film thickness is of main importance for the optical transducer detected by Surface Plasmon Resonance (SPR) what is used in our study.

Hundreds of immobilization protocols have been developed in an effort to ensure high performance sensing. All of them are focused on finding and deposition of appropriate matrices in which the recognition medium can be incorporated. However, the matrix always deteriorates the effectiveness of recognition. It seems that the best approach is to perform direct immobilization of the recognition medium. However, this is not always possible regarding the organic materials – the problem is whether the deposition retains the bioactivity of the recognition agent. On the other hand, the type of the transducer also imposes constrains. For example, the direct immobilization of the proteins is not possible for electrochemical sensors. Evaluating the pros and cons of organic (protein) film deposition we have considered the possibility for direct immobilization of Hb and Mb on SPR transducer. To best of our knowledge, SPR biochip with immobilized Hb and Mb has never been constructed before. We have used spin coating, for direct immobilization and MAPLE for elaboration of the SPR biochip.

The performance of both SPR chips – direct and MAPLE immobilized, was studied by SPR registration of the binding activity of Hb and Mb ligands with carbon monoxide (CO), carbon dioxide (CO2) and nitride oxide (NO).

2. Deposition techniques

2.1 Spin coating

Spin coating has been used for direct immobilization of recognition agents. An appropriate solvent nd right concentration of the recognition agent was established. The concentration is related to establishing spin speed and duration for obtaining required layer thickness what is very important for providing SPR detection with maximum accuracy and sensitivity. As a transducer we have used a gold covered diffraction grating. Experimentally we have found that the optimal layer thickness is 100 – 150 nm for the applied concentrations of the recognition agents. The solvent for Mb and Hb is deionized water. The obtained layers have been very uniform and with good optical quality.

2.2 MAPLE technique

Mb and Hb are dissolved in a solution of a volatile matrix (polyethylene glycol, PEG). The deposition occurs in a vacuum chamber under the irradiation of UV light. Before the deposition starts the solution is frozen. The idea of the technique is that the matrix absorbs the laser light, so that decomposition of the recognition agent is avoided.

In our experiment, PEG was chosen as a matrix because of its well-expressed absorption at 355 nm. This coincides with the irradiation that we apply – the third harmonic at 355 nm of Nd:YAG laser. The pulse duration is 10 ns, the repetition rate 10 Hz and the controllable energy is between 1 mJ and 10 mJ. The laser irradiation is focused with a quartz lens with focus length 50 cm. The laser fluency of our experiments varied from 100 mJ/cm2 to 500 mJ/cm2. During the deposition process the laser light scans continuously the target by managing the tilt of a control mirror. The target is frozen at temperature between 15 C – 20 C. The substrate temperature is not controlled - during the deposition it is in the range 25 C – 32 C. The vacuum is about 2.10-5 mbar, achieved by using standard rotary and turbomolecular pumps.

2.3 Layer's properties

The deposition of layers was performed simultaneously on glass substrate and on a diffraction grating covered with a gold layer. The glass substrate is with optical quality 40 -10, that permits to measure the layer thickness with accuracy up to 10 nm. The layer thickness was measured by 3D Optical Profiler Zeta 20 and by Talystep Filmetrics F20. We are able to control the layer thickness in the range 100- 150 nm for both spin coating and MAPLE deposition.

3. Results and discussion

3.1 Layer's properties

Fig. 1a shows a SEM picture of a Hb layer with thickness 110 nm. It shows that on the relatively uniform background there are particles with regular and irregular form. The SEM shows that they have spatial or flat shape. We have found that their quantity and shape strongly depend on the pulse energy and on the distance to the target, while the background was influenced in its density and hardness. Obviously, bigger particles come from PEG during the deposition. Unfortunately, SEM cannot resolve the background structure, but based on our measurements, addressing the Hb functionality, one can conclude that this is a Hb layer. Similar observations and conclusions are made for the Mb layer.

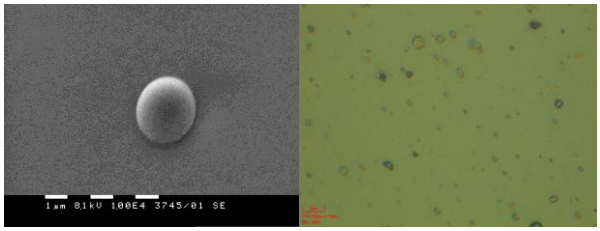


Fig 1(a). *SEM pictures of a deposited Hb layer Figure 1 (b). An optical microscope picture of the same layer*

The thickness uniformity of the layer is in the framework of the accuracy of the measurement – 10 nm, what is rather a good result for MAPLE deposition. This is confirmed by SPR excitation at different points on the gold diffraction grating having dimensions 16x11 mm. The resonance shift was no more than 2 nm. Regarding spin coated layers, the uniformity and optical quality is very good.

3.2 SPR excitation and registration

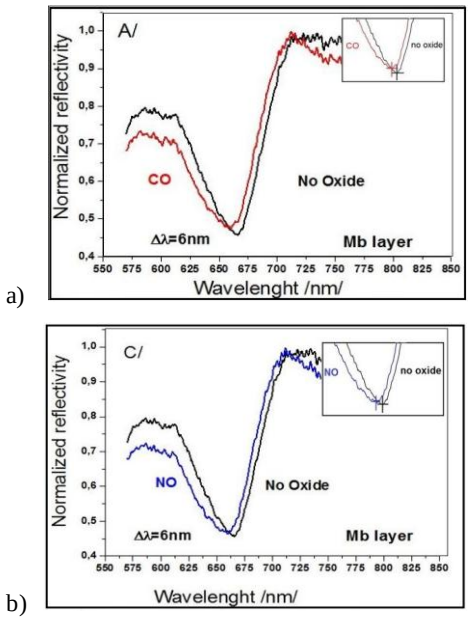
The transducer is a gold diffraction grating. The spectral readout is used for SPR excitation and registration. White light under angle of incidence in the range 35 – 42 degrees excites resonances between 708 nm and 610 nm for a bare grating. The angle of incidence is controlled with accuracy 0.01 deg. The spectrometer registers the spectrum in the zero order reflection. SPR is measured with accuracy of 3 nm.

3.3 Functionality of the Mb and Hb layers

The functionality of Mb and Hb consists of the functionality of their various functional groups. It can be examined by checking the affinity for the ligand of interest. Besides the oxygen, Mb and Hb ligands also include CO, CO2 and NO that bind to different functional groups. The binding reaction has been registered by SPR, proving the existence of corresponding functional group and respectively demonstrating the functionality of the Mb and Hb deposited layer. The functionality of layers deposited by spin coating and MAPLE technique has been examined.

3.4 Functionality of spin coated layers

The affinity of iron in the porphyrin core towards CO is very high for Mb and Hb and it displaces the oxygen bound to the iron atom. This reaction has been clearly observed blowing the Mb/Hb layer, deposited on a gold diffraction grating, with CO having concentration 1000 ppm. The resonance shift as a results of the CO binding is about 6 nm, shown in Fig.2a



a)

b)

Figure 2. *SPR detection of the binding activity of different functional groups of Mb layers*

The registration of CO2 binding proves the presence of small amount of corresponding functional groups, in this case – an amino group of the globin protein. Despite the binding activity is much lower than the one to CO, the effect is well pronounced. The resonance shift is about 6 nm showing that the functional group is "alive" and keeps its activity.

The same conclusion can be made for thiol groups in the globin protein which are responsible for NO binding (Fig.2b). Comparing the binding activity of CO and NO, the latter is higher – their resonance shifts are compatible, despite that the NO concentration is five times lower (200 ppm). It is worth mentioning that we have registered activity of all samples that we have prepared by spin coating, but the sensitivity (namely the activity) of the deposited layers was different despite the deposition parameters were exactly the same. Here we present the highest activity we have achieved.

3.5 Functionality of MAPLE deposited layers

The problem about repeatability regarding bioactivity is more serious for MAPLE deposited layers. Fig. 3 proves the activity of heme and thiol groups of the Hb layer after deposition and shows the most expressive SPR shift for CO binding and for NO binding.

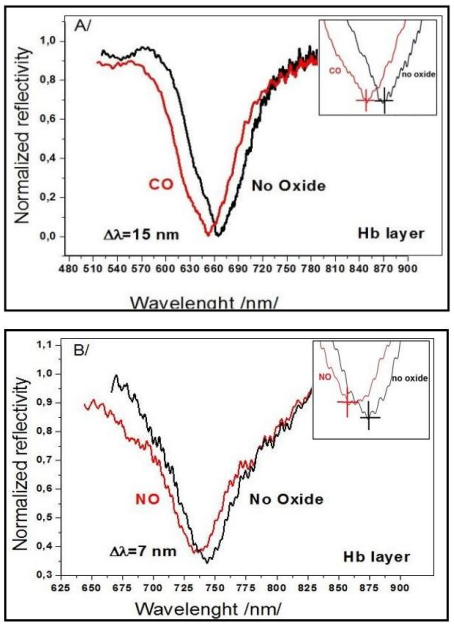


Figure 3. *SPR detection of the binding activity of a Hb layer deposited by MAPLE technique*

Despite the illustrated activity, more pronounced that in the case of a spin coated layer, we cannot argue that MAPLE deposition is more effective. The problem is that it was not possible to get the same results although we followed the same technology recipe

Probably, we face the problem related to the abundance of particulates of regular or irregular shape, deposited by MAPLE. This could alter seriously the biological activities of proteins as far as it is related to their three-dimensional structure. The structural alterations of proteins, probably depending on aggregation during the deposition, frequently result in the loss of the activities.

4. Conclusion

We have shown that myoglobin and hemoglobin can be deposited directly by spin coating and by MAPLE technique keeping their functionality. The functionality has been proved by SPR detected binding of CO, CO_2 and NO to the corresponding functional groups of the proteins. The deposition has been performed by one step and in one technological cycle, which is extremely useful.

It is difficult to access which deposition method is more effective but for both methods the tide control of the technology parameters is very important.

In fact, we have made gas sensors based on heme proteins with SPR transducer, that is reported for the first time, to the best of our knowledge.

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