Alternative technique for direct immobilization of biomolecules

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Abstract. The conventional approach to immobilization of biomolecules on the transducer of biosensors requires their embedding into the so-called embedding matrix. The procedure is complicated and laborious and performed by chemical means. As a result, not only biomolecules, but also other substances are deposited on the desired surface. This leads to reduced sensitivity and specificity due to the non-specific reactions with the molecules of the embedding matrix. Immobilization without embedding matrix (the so-called direct immobilization) guarantees the specificity of the biosensor and increases the sensitivity and accuracy. The main challenge in implementing this strategy is to preserve the bioactivity of the ligand. We have successfully solved the problem by using the matrix-assisted pulsed laser evaporation (MAPLE) technique. Here we report the properties of hemin directly immobilized on the transducer of a biosensor based on surface plasmon resonance (SPR).

1. Introduction
The surface plasmon resonance (SPR) is a detection method providing high accuracy and real-time registration. In terms of specificity, it is inferior to label-based detection methods due to a bimolecular reaction initiated by the molecules of the embedding matrix.

The recognition molecules (ligand) are deposited on the surface of an SPR chip (in our case a gilded diffraction grating). Proteins and other biomolecules decrease sufficiently their bioactivity after deposition by conventional methods on a gold surface [1].

We have shown that MAPLE is a very effective method of ligand deposition on an SPR chip. In [2], we analyzed the properties of MAPLE deposited layers of hemoglobin and myoglobin and showed that intact molecule deposition is achieved. In [3], a record sensitivity is demonstrated in glucose detection with hemoglobin as a ligand. The results show that using MAPLE preserves the biological activity, increases the sensor sensitivity and provides good adhesion between the gold surface and the recognition element, which makes the repeated use of the SPR chip possible.
2. The biorecognition element and the SPR chip

Hemin (an iron-containing porphyrin) is a red-brown to blue-black crystalline salt \( \text{C}_{34}\text{H}_{32}\text{N}_{4}\text{O}_{4}\text{FeCl} \) derived from oxidized heme but usually obtained in a characteristic crystalline form from hemoglobin.

Hemin is not as sensitive to the immobilization process as the three-dimensional proteins, such as hemoglobin and myoglobin. Moreover, it possesses a high potential as a recognition element for the detection of sulfides, hydrogen peroxide, nitrite, dopamine and DNA [4]. This makes it extremely attractive in the manufacture of biosensors, provided the main difficulty associated with is overcome, namely, its poor solubility in both organic and aqueous solvents [5]. So far, its application has been limited to electrochemical sensors in compound due to its low solubility. Typically, hemin is used in combination with other substances by embedding it in a matrix structure [6].

In the work reported, hemin was deposited on an SPR chip – a gilded polycarbonate substrate for a CD-R disk. Before the MAPLE deposition process, the grating was immersed in isopropyl alcohol and cleaned ultrasonically.

We show that the direct immobilization of hemin by MAPLE preserves its bioactivity. This was proved by registering the intermolecular reaction between nitric oxide (NO) and the layers of hemin deposited on the surface of the SPR chip. The details about the SPR set-up and registration can be found in [2].

3. MAPLE

The MAPLE process is performed in a high-vacuum environment. The recognition elements are dissolved in a solvent absorbing the laser light that is subsequently frozen to form a solid target. In this way, the violent interaction between photons and biomolecules is diminished since the main fraction of laser energy is absorbed by the solvent.

The deposition on the substrate chosen takes place in a vacuum chamber, as shown in figure 1. The pressure is about \( 2 \times 10^{-5} \) mbar; the laser wavelength is 1.06 µm. During the evaporation, the target temperature is in the range between \(-40 \degree C\) and \(-70 \degree C\). The pulse duration is 10 ns at a repetition rate of 10 Hz. The laser fluence is varied from 80 mJ/cm\(^2\) to 120 mJ/cm\(^2\). During the deposition process, the laser beam scans continuously the target. The distance between the frozen target and the SPR chip is 10 cm.

Regarding the deposition of proteins as recognition elements, in our previous work we found that the MAPLE parameters should be varied in accordance with the molecular weight and the conformational state of the deposited molecules [2]. Therefore, precise adjustment of these parameters is required.

![Figure 1. MAPLE technique set-up.](image)
4. Results

4.1. Deposition of hemin
For layer deposition, 10 mg hemin (bovine) was dissolved in 1 ml of 5% deionized water solution of triethylamine. The solution was filtered through a 600-nm pore polyether sulfone membrane filter, and then frozen in the vacuum chamber at −40 °C. Deposition was performed not only on an SPR chip, but also on Si substrates and on standard copper TEM grids in order to analyze the layer thickness and morphology.

4.2. MAPLE deposition effectiveness
The effectiveness of the MAPLE deposition is defined by the degree of bioactivity of the immobilized ligand. The bioactivity of MAPLE-deposited hemin was assessed by SPR registration of nitric oxide (70 ppm) binding with the hemin. Figure 2 shows the SPR spectral shift, amounting to about 3 nm, resulting from the nitric oxide (NO) – hemin reaction. The effect is sufficiently well expressed, despite NO being a very small molecule. This proves the bioactivity of the deposited hemin, as well the high sensitivity of the SPR detection.

![Figure 2. SPR shift resulting from NO binding to a MAPLE-deposited hemin layer (140 nm).](image)

4.3. Layer analysis
The thickness of the hemin layer was about 140 nm. It strongly depends on the number of laser pulses, while its uniformity depends on the distance between the frozen target and the substrate. The high sensitivity of NO detection and the AFM image in figure 3 both show that MAPLE parameters are optimally balanced to deposit uniform film of the hemin bioactive molecules.

![Figure 3. AFM image of MAPLE-deposited hemin layer on a Si plate; the deposited layer boundary is clearly visible.](image)
The SEM study of the hemin layer revealed its morphology. It consists of chain-arranged hemin crystals with a size of approximately 1 µm, as shown in figure 4. This confirms that the MAPLE deposition has been performed very “gently”, without distorting the hemin molecules – after evaporation they maintain their natural crystalline form. It is remarkable that no trace of trimethylamine is seen in the deposited layer.

![SEM image of hemin layer deposited by MAPLE.](image)

**Figure 4.** SEM image of hemin layer deposited by MAPLE.

As in the case of hemoglobin, we found that the biological functionality of the deposited hemin layer decreases over time. The question is: can trimethylamine stabilize hemin’s bioactivity if it is deposited in the layer, even at the expense of the level of bioactivity? In order to check this hypothesis, we studied the absorption spectra of a hemin solution over time. Figure 5 shows the absorbance spectra in the UV VIS range of a hemin solution measured immediately after preparation and 15 days later. The absorption peak at 384 nm, which is characteristic of hemin, is observed in both measurements, but the absorption decreases over time, which is associated with decreased bioactivity. Thus, as for all biomaterials, the bioactivity of hemin is also time limited.

![Absorbance spectra of a hemin solution – on the day following preparation and 15 days later.](image)

**Figure 5.** Absorbance spectra of a hemin solution – on the day following preparation and 15 days later.

5. Conclusions
In the present work, we demonstrated a direct immobilization of hemin performed by the MAPLE technique. A layer of hemin having a pre-defined thickness and good uniformity was deposited on an SPR chip. The high detection sensitivity and, respectively, the high bioactivity of hemin molecules was assessed by SPR measurements. Regarding the decreased biological activity of the layers, it was concluded that could not be improved by trimethylamine if it is also deposited in the layer. The MAPLE
parameters should probably be subjected to additional optimization in view of achieving a higher bioactivity. As a conclusion, directly immobilized hemin opens new possibilities for application in biosensors.

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