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FUNCTIONALIZATION OF HIS-TAGGED HUMAN KAPPA OPIOID RECEPTOR ON THE GOLD SURFACE OF CANTILEVER ARRAYS

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ABSTRACT
Biosensors are promising devices for detecting interactions between both chemical and biological molecules and exploring a novel molecule. In this study, one-end-fixed and doubly-clamped micron-sized nickel cantilevers with integrated diffraction gratings are fabricated and actuated magnetically by applying AC signal to an electromagnetic coil to be used as resonant mass biosensors for detecting narcotics in bodily fluids. To achieve this goal, we have cloned and expressed human kappa opioid receptors (hKOR) in *E.coli.* Then, we immobilized opioid receptors via thiol groups on gold surfaces of the cantilevers. Our results indicated that we have successfully functionalized protein on the gold surface and the amount of protein was measured as 85 pg by a photodetector (PD) setup.

KEY WORDS
Human kappa opioid receptors (hKOR), biosensor, and optical readout

1. Introduction

Biosensors are powerful devices to investigate molecular interactions between a molecule introduced in a liquid solution and a solid support immobilized on the surface. They can be used for monitoring interactions of proteins as well as characterizing biochemical processes. Clinically interesting proteins in human serum can be also detected [1]. Hence, biosensors will be the essential tool in clinical, criminal and research bases.

The immobilization of biomolecules as solid support is extremely important to provide responsive surfaces where ligands will be introduced. By optimizing immobilization of biomolecules, ligands to be detected are able to interact with immobilized molecules without any steric restrictions [2]. One method to achieve this is the oriented immobilization of proteins. In this method, tagged proteins interact non-covalently with the complex immobilized on the surface [3,4].

The Ni-NTA/His-tag system has recently become a popular tool for functionalization of biosensing surfaces [5-10]. There are six binding sites in the structure of Ni-NTA. Four out of six binding sites are shared by Ni^{+2} and the rest of the sites are filled with two histidines [2,6]. Hence, inserting a hexahistidine extension from a specific position of the protein enables constituting a his-tagged protein that can be readily bound to the immobilized complex [6].

In this study, hexahistidine tagged hKOR was generated using the PCR method. Chimeric proteins were expressed in *E.coli*. After the purification of hKOR, they were immobilized on a gold coated cantilever array of a resonant mass biosensor via thiol groups and a Ni-NTA complex. Then, the presence of proteins on the cantilever surface was detected using a PD setup in the amount of 85 pg. Such an approach may utilize to detect narcotics in bodily fluids.

2. Methods

2.1 Cloning of hKOR

The cDNA of hKOR was PCR amplified from pcDNA1 vector. PstI site and 6 x His-tag were obtained by the following primers: Forward 5' - AGAGTCGACC TGCAGATGGACTCCCCGATCCAGATCTTC - 3' and reverse 5' - CAAGCTTGCCTGCAGCTAGTGGTGGT GGTGGTGGTGTACTGGTTTATTCATCCC - 3'. The amplified receptor was cloned to pMAL-c2x vector and transformed to DH5α.

2.2 Expression and Purification of hKOR

Overnight culture was diluted 1:100 and inoculated in LB supplemented with 100mg/ml of ampciline. Protein expression was induced at OD_{600}=0.6 with 0.5mM IPTG for 9 hours at 37^0C. Cell culture was collected by centrifugation at 5000g for 10min. The pellet was treated with 10mM Tris-HCl, pH 8 supplemented 1mg/ml of lysozyme for 30min at 4^0C. Further degradation of the bacterial cell wall was obtained by sonication for 5x30sec. The cell lysate was centrifuged at 1000g for 15min at 4^0C to remove unbroken cells and the supernatant was further centrifuged at 10000g for 30 min at 4^0C. Then, the pellet was treated with solubilization buffer containing 100mM NaH_2PO_4, 10mM Tris-HCl, 20mM β-mercaptoethanol, pH 8 and 0,1% of SDS and solubilization of hKOR was allowed for 1 hr at room temperature with gentle shaking. After centrifugation at 20000g for 30min, hKOR proteins

were incubated with Ni-NTA resin, which was prequilibriated with the solubilization buffer, for 1hr at room temperature. The MBP-hKOR-6XHis was eluted with solubilization buffer containing 300mM imidazole. The eluted protein was dialyzed against 100mM NaH_2PO_4, 10mM Tris-HCl, pH 8, 0,1% SDS (w/v) and 50%Glycerol (v/v) for overnight at room temperature to be stored at -20^0C.

2.3 Western blot analysis of hKOR

After SDS-PAGE with 10% polyacrylamide gel was performed; separated proteins were transferred to nitrocellulose membrane for western blot analysis. The proteins were detected by anti-his probe (1:750) and AP conjugated anti mouse IgG (1:10000).

2.4 Immobilization of hKOR on a gold surface

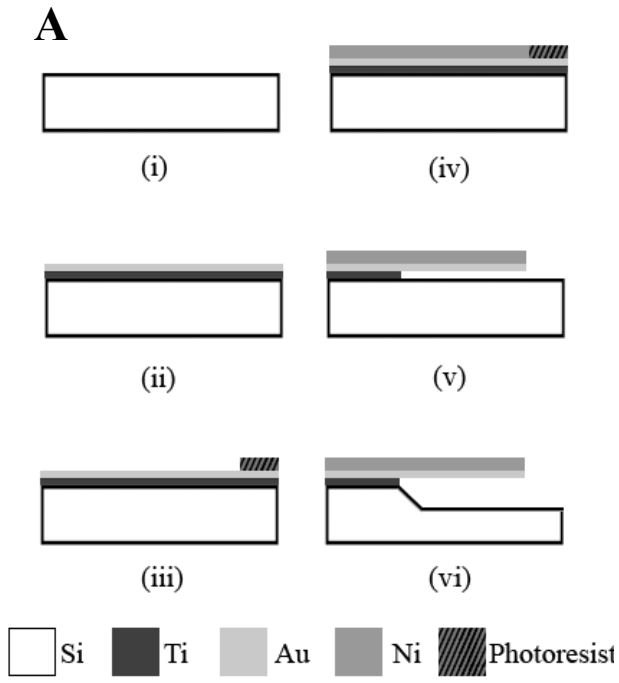
Thiol groups were formed on the gold surface as in [10]. The protein was allowed to be immobilized on gold surface, which was equilibrated previously with the solubilization buffer without β-mercaptoethanol, for 1hr with gentle shaking. Then, the gold surface was blocked by 5% BSA for 1 hr to avoid any non-specific protein binding. To verify immobilization of proteins on gold surfaces, a 5x5mm gold coated test sample was treated with anti-his probe (1:750), and subsequently with FITC conjugated anti mouse IgG (1:750) for 1hr after immobilizing the proteins. Then, the test sample was examined under fluorescence microscopy with 10x magnification. All processes were held at room temperature.

2.5 Detection of Opioid Receptors

The detection mechanism is based on the measurement of the shift in the resonance frequency. Each cantilever has a unique resonance frequency which is a function of its mass and stiffness. Once the mass changes due to the accumulation of species on the cantilever, the resonance frequency also changes. This change is then directly related to the change in mass.

In our approach a diffraction grating was integrated to the tip of each cantilever for interferometric measurements. Using a PD setup the resonance frequency could be picked up with good accuracy. In fact, a comparison of the PD measurements with those obtained on a commercial Doppler vibrometer yielded errors less than half a percent. Furthermore, PD measurements were observed to be immune to environmental noise as opposed to vibrometer measurements which required vibration isolation.

The fabrication of the sensor was based on a single-step photolithography followed by electroplating and release (Fig. 1). A <100> Si wafer was first coated with 10nm thick Cr and 100 nm thick Au layers by sputtering. These would serve as the seed layer for the subsequent Ni plating. After the Au surface was patterned with lithography, Ni plating was carried out in a conventional nickel sulphamate bath [11]. A variety of cantilever widths from 4 to 10 μm were used. Cantilever lengths were taken to be ten times the width. Formation of cantilevers by plating was followed by the removal of Au and Cr from the Si surface and release of the cantilevers in a 35% KOH solution at 80 °C, where Ni served as a natural mask. Etching of Au was carefully timed to avoid removal of the Au coating from beneath the cantilevers for further functionalization.



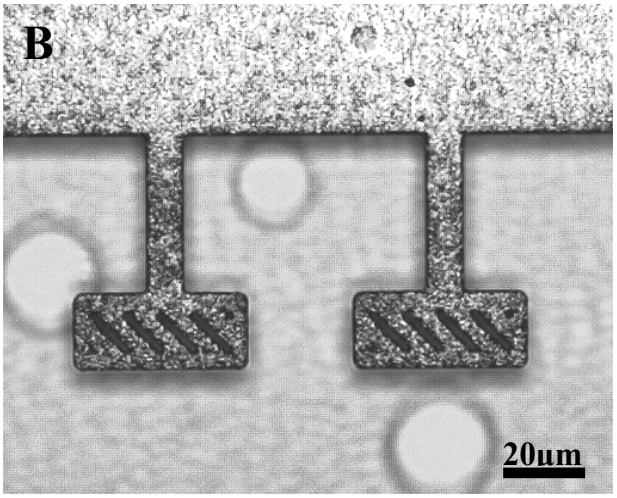


Figure 1. A. Fabrication flow: (i) Standard wafer cleaning. (ii) Sputtering of Ti /Au (10nm/100 nm) (iii) UV-Photolithography. (iv) Electroplating of Ni as structural layer (v) Photoresist strip and etching of Au and Ti layers (vi) Anisotropic Silicon etching. B. An array of fabricated cantilevers with a thickness of 0.7μm and with a platform of four 3 μm-period diffraction gratings.

For resonance frequency measurements grating interferometry was used as indicated above. Cantilevers were actuated by an external magnetic coil. A laser source was used to illuminate the grating, which served as the measurement platform, whereas Si surface below was used as the stationary reference. The intensity of the diffracted light changed periodically as a function of the distance from the cantilever to the Si surface. Hence, monitoring of this modulation via PD lead to the direct measurement of the frequency. The point where the maximum peak-to-peak voltage was obtained was considered as resonance.

3. Results and Discussion

3.1 Expression and Purification of hKOR

hKOR expression was optimized by taking samples at every 3 hr. The optimum expression level was found to be highest after 9hrs (data not shown). The purification of recombinant proteins was performed by Ni-NTA resin. Then, stability and intactness of protein were controlled by the Western Blot analysis. The apparent molecule weight of hKOR is around 85kDa with fusion protein MBP (~40kDa) as shown in Fig 2. This indicates that the protein is intact and stable under our purification system.

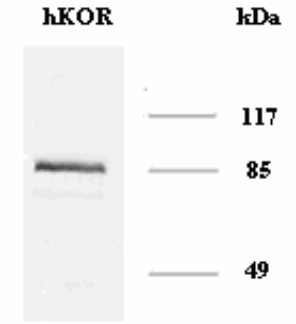


Figure 2. Western blot analysis of purified hKOR. Histidine tagged hKOR is at 85kDa with fusion protein MBP.

3.2 Immobilization of hKOR on gold coated cantilever array

hKOR proteins were immobilized on the surface of the cantilevers manufactured by [12]. First, DSP and Lysine-NTA were reacted to form a functional group to be able to crosslink protein on the surface. The complex was added on the surface followed by the addition of $NiCl_2$ and the protein. To see whether proteins were immobilized on the surface, the antibody that recognizes the His tag was cross-reacted with the gold surface. Then, the secondary antibody, which is FITC-conjugated, was added on the surface to detect the primary his-tag antibody. Immobilization of proteins on the gold surface is observed using fluorescence microscopy as shown in Fig. 3A where bright spots represent immobilized protein molecules. No signal is observed at the blank surface (Fig. 3B), which indicates that antibodies do not bind non-specifically and signals observed at Fig 3A are possessed by immobilized protein molecules.

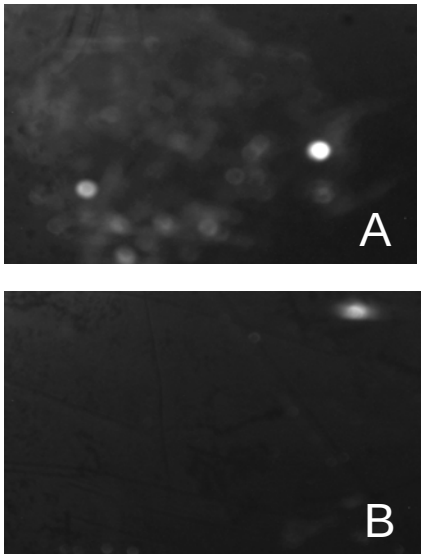


Figure 3. Fluorescence microscopy results of immobilized proteins with magnification of 10x. A. Immobilized hKOR on gold surface treated with FITC conjugated IgG (1:250) B. Blank gold surface treated with FITC conjugated IgG (1:250).

3.3 Detection of Opioid Receptors

The results of frequency measurements prior and after the functionalization of a 7 μm-wide cantilever with a length of 70 μm are given in Fig. 4. The total shift in the frequency amounts to 180 Hz which corresponds to a mass accumulation of 85 pg. A monolayer of the proteins on the specific cantilever is estimated to weigh 40 pg. Since hKOR is a membrane protein, micelle formation occurs during purification; hence, an additional mass accumulation is generated.

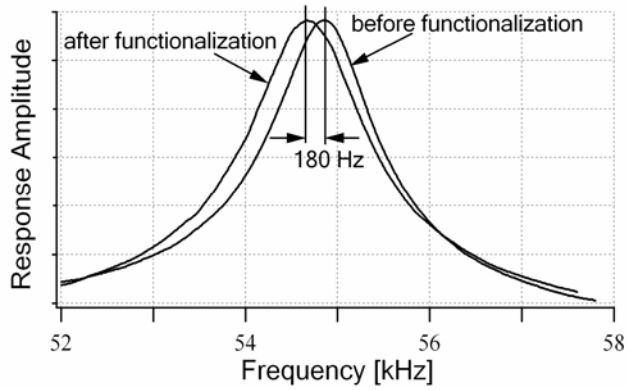


Figure 4. The shift in resonance frequency of a 7x70 μm cantilever upon exposure to opioid receptors.

Of course the study did not address the selectivity issue, since the gold surface could act as a functionalization platform for a variety of other molecules. The emphasis was rather placed on i) demonstrating the operation of the grating interferometry and ii) attaching the opioid receptors on cantilevers prior to further exposure to drug ligands. The same measurement principle would then be employed to detect and quantify drug ligands.

4. Conclusion

This study indicated that we successfully immobilized proteins on the cantilever surface and the detection of proteins on the cantilever surface was achieved by a PD setup. This device may be used for the detection of narcotics from biological samples and other biological applications.

Acknowledgements

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