Immobilization of His-Tagged Human Kappa Opioid Receptor on a Cantilever Array Biosensor for Detection of Narcotics in Bodily Fluids

by

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This is to certify that I have examined this copy of a master's thesis by

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and have found that it is complete and satisfactory in all respects,

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ABSTRACT

Biosensors are promising devices for detecting interactions between both chemical and biological molecules and exploring a novel molecule. The immobilization of biological molecules on surfaces has a crucial role in the fabrication of biosensors. This study presents functionalization of human κ opioid receptor (hKOR) for a novel biosensor application to detect drug molecules from human bodily fluids. For this purpose, the receptor was first cloned to expression vectors and expressed in *Escherichia coli.* Then, recombinant hKOR was purified by affinity chromatography. For the first time, the purified receptor was immobilized on the micro-fabricated cantilever array biosensor via thiol groups and Ni-NTA complex. The immobilization of the receptors was verified with fluorescence microscopy. A specific and label-free detection of interaction between hexa-histidine tagged hKOR and his-antibody has been demonstrated.

ÖZET

Biyosensörler, kimyasal ve biyolojik moleküller arasındaki etkileşimleri gözlemlemek ve yeni molekülleri bulmak üzere kullanılabilecek önemli cihazlardır. Biyolojik moleküllerin yüzeye sabitlenmesi, biyosensör üretiminde krtitik rol oynamaktadır. Bu çalışmada, insan κ opioid almaçlarının (hKOR) biyosensör alanında yeni bir uygulama olan insan vücut sıvılarından uyuşturucu tayini yapılması amacıyla yüzey işlevselleştirilmesi gerçekleştirilmiştir. Bu amaçla, almaçlar ilk olarak ifade vektörlerine klonlanıp *Escherichia coli*'de ifade ettirilmiştir. Daha sonra, rekombinant hKOR affinite kromatografi yöntemi ile saflaştırılıp, biyosensör yüzeyine tiyol ve Ni-NTA grupları aracılığı ile sabitlenmiştir. Başarıyla yüzeye sabitlenen almaçlar flurosan mikroskobu ile görüntülenmiştir. Altı histidin ile işaretlenmiş hKOR ve histidin antikoru arasındaki protein- antikor etkileşimi doğrudan ve etiket gerektirmeyen bir yöntemle gösterilmiştir.

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Chapter 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, 2]. Hence, biosensors will be the essential tool in clinical, environmental, industrial and research bases.

The immobilization of biomolecules on a 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 [3]. 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 [4, 5].

The Ni-NTA/His-tag system has recently become a popular tool for functionalization of biosensing surfaces [3, 5-12]. 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. 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.

In this study, hexa-histidine tagged human κ opioid receptor (hKOR) was generated using the PCR method. After the receptor was cloned to expression vectors, it was expressed in *E.coli*. The purification of hKOR was performed and the receptor was immobilized on a cantilever array biosensor via thiol groups and a Ni-NTA complex. Then, the presence of proteins on the cantilever and the specific interaction with anti-his antibody was detected using a label-free method. Such an approach may utilize to detect narcotics in bodily fluids.

Chapter 2 provides necessary background and literature review on biosensor technology, surface functionalization techniques and human opioid receptors. The biosensor applications and previous studies on immobilization of his-tagged proteins are reviewed.

Chapter 3 describes the methods for cloning of human opioid receptors into expression vectors, expression and purification of these receptors together with immobilization to the cantilever array biosensor and assessing bio-sensitivity and bio- selectivity of the sensor.

The results are given in Chapter 4, together with the discussion of these results.

The thesis is concluded with a short summary of the performed study and future research work.

Chapter 2

LITERATURE REVIEW

2.1 Biosensors

A biosensor is a device detects and/or measures an analyte by converting a biological response to an electrical signal. It consists of three parts: Biological recognition element, a transducer and signal processors (Figure 2.1).

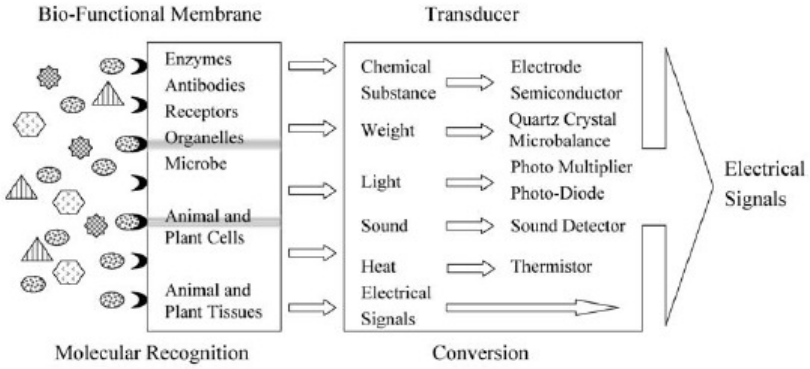


Figure 2.1 The principle of a biosensor. [13]

Biosensors have wide variety of applications and some are commercially available. The first biosensor was developed in 1962 for monitoring oxygen levels in liquids [14] and released in market in 1975 by Yellow Spring Instruments Company to detect glucose in blood. Up to date, many biosensors have been developed for monitoring the quality of

foods [15], waste and environmental water [16], presence of pollutants, toxins and pesticides [16], identifying novel drugs [17], detecting pathogens [18,19], mutations on nucleic acids [20-22] and clinically important markers [1,2,23] for early diagnosis of diseases.

2.2 Surface Functionalization for immobilization of His-tagged Proteins

2.2.1 Surface Chemistry

Attachment mechanisms for capturing of ligands can vary. A ligand can be attached and be immobilized onto a surface in different chemical ways. The four common ways of ligand attachment mechanisms are Electrostatic/Hydrophobic interaction, Physical Entrapment, Covalent Binding and Biorecognition oriented [4] . In Figure 2.2, a general classification of ligand attachment mechanisms is demonstrated.

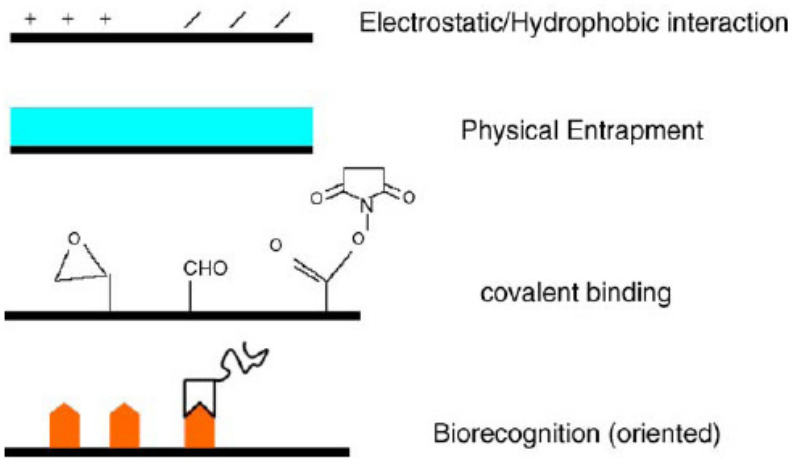


Figure 2.2 Attachment mechanisms for capture ligands. [4]

The attachment of a ligand to a surface via electrostatic or hydrophobic interactions is a straightforward and well established [4]. The ligand is simply adsorbed on the surface via electrostatic or hydrophobic forces. Despite the simplicity of this mechanism, there are several issues should be pointed out. Since these forces are weak, the ligand can be detached from the surface after extensive washing. Moreover, non-specific binding of proteins is also known and proteins can be denatured on a hydrophobic surface as well [4].

Another attachment mechanism is to capture ligands by physical entrapment. In this mechanism, ligands are captured in a polymeric interface. The proteins are not crosslinked to the surface. This polymeric interface enables one to achieve controlled distribution of proteins without any denaturation problem [6]. Since the surface is coated with the polymer, the non specific binding is not observed [6]. However, the response time of the biological interaction can be prolonged due to diffusion limitations [4].

The covalent binding of proteins is more robust attachment mechanism. In this mechanism, a crosslinker is generally used to have active groups on the surface where analytes can bind. Although proteins are attached to the surface strongly, they may unfold.

Bio-oriented immobilization is the only attachment mechanism that proteins are immobilized on the surface in an orientation dependent way [4]. In this mechanism, the molecules to be detected are labeled with specific tags to bind non-covalently to the interaction partner of the tag. Generally used tags and their interaction partners are: biotin–avidin, Ni-NTA & His-tags and glutathione & GST tags. Although the probe molecule should be tagged, this approach provides oriented and uniform immobilization of proteins [4].

2.2.2 Surface Functionalization with NTA (N- nitrilotriacetic acid)

In the development of biosensors, the immobilization of biomolecules at interfaces plays a crucial role. To obtain highly sensitive sensing surfaces, it is needed to introduce a receptor molecule. By this way, the corresponding ligand can be bound without steric restrictions. One method to achieve this is the biorecognition oriented immobilization of proteins, ideally at a site which does not interfere with the ligand-binding domain. [3,5-12]

The NTA/His-tag system [24] has become a powerful and popular tool for the one-step purification of His-tag proteins. Incorporating penta or hexa histidine tag in the protein enables the attachment of the target protein to the immobilized complex for purification. The NTA forms a complex with divalent metal ions such as Ni^{+2}, Co^{+2}, Cu^{+2}, or Zn^{+2}. Divalent metal ions interacts with four of the six binding sites in the complex. Remaining two binding sites can be occupied with electron donor groups as imidazole ring on histidine residues (Figure 2.3). The electron donor groups that occupies the two remaining sites of the complex should have enough flexibility to fit the complex. The His-tag has enough flexibility to bind to both unoccupied sites of the complex [25]. The non specific binding of proteins to the immobilized complex is not observed since a stable complex between the immobilized complex and his tag of the protein with the stoichiomethic ratio of 1:2 and the 1:1 ratio of histidine:complex is not stable [26]. Moreover, adsorbed proteins can be easily desorbed by the addition of a chelating agent as ethylene diamine tetraacetic acid (EDTA) or a competitor such as imidazole, or by acidification. [3,5-12]

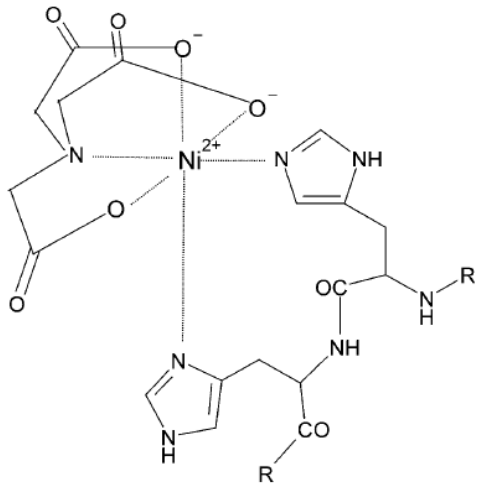


Figure 2.3. Interaction of two histidine residues with Ni^{+2}-NTA. [7]

Recently, researchers started to exploit the principle of NTA/His-tag purification system for sensor and protein array technology. Ni^{+2}-NTA has been used as the basis of protein deposition on optical [6,8,9,12], and gold [3,7,10] surfaces and electrodes [9,11] using self-assembled monolayers or polymeric interfaces [6] for applications in immunosensors [9], protein arrays [6,8] and sensor chips [12]. A commercial sensor, BIOCORE, is readily available in the market utilizing the Ni-NTA functionalization for sensing his-tagged proteins [12].Throughout these studies, the NTA/His-tag system was well characterized with different proteins using surface plasmon resonance (SPR), fluorescence microscopy, and atomic force microscopy (AFM) . It is observed that his-tagged proteins are adsorbed rapidly on the NTA functionalized surface. Moreover, it is discovered that non-specific binding does not occur and surface can be regenerated. The system also allows both label and label free of detection of proteins. Hence, this attachment mechanism enables one to develop a sensitive, selective, stable, reproducible and cost-effective system for biosensor and protein array technology.

2.3 Human Opioid Receptors

Human opioid receptors are G-protein coupled receptors that regulate homeostatic functions and reward mechanism of the brain through neurotransmitters and hormones [27-29]. Activation of these receptors leads to initiation of complex cellular processes [29]. There are four major types of opioid receptors cloned: DOR [30-31], MOR [32-34], KOR [35-38] and NOR [39]. The names of κ and μ receptors were derived from the first letter of their first discovered ligands as ketocyclazocine for κ receptor (KOR) and morphine for μ receptor (MOR). On the other hand, δ receptor (DOR) was named after mouse vas deferens where it was first identified. Recently, a new type of opioid receptor was discovered and named as the nociceptin receptor (NOR) or ORL 1 receptor. Other types of opioid receptors were also proposed: ε-, ι-, λ- and ζ-receptors, however, they were poorly characterized [40]. The σ-receptor was also classified as an opioid receptor when it was first discovered, but, it is no longer identified as a member of opioid receptors since it does not share the common characteristics of opioid receptors such as the stereoselectivity and antagonism [40].

Opioid receptors are distributed in central nervous system as well as periphery. Each receptor are localized at different tissues to serve different functions upon activation. The location and function of opioid receptor are summarized at Table 2.1

Table 2.1 Location and function of opioid receptors [41,42]

Opioid receptor	Location	Function
DOR	Brain: - Pantine nucleus - Amygdale - Olfadey bulbs - Deep cortex	- Analgesia - Eupheria - Physical dependence
KOR	Brain: - Hypothalamus - Periaqueductal gray - Claustrum Spinal Cord: - Substantia gelatinosa Peripheral nervous system	- Spinal analgesia - Sedation - Miosis - Inhibition of antidiuretic hormone release
MOR	Brain: - Thalamus - Periaqueductal gray - Laminae İİİ and IV of the cortex Spinal Cord: - Substantia gelatinosa Peripheral nervous system	- Supraspinal analgesia - Physical dependence - Respiratory depression - Miosis - Euphoria - Reduced gastrointestinal motility
NOR	Brain: - Cortical areas - Olfactory regions - Limbic structures - Thalamus - Central periaqueductal gray - Substantia nigra, - Several sensory and motor nuclei Spinal Cord Peripheral nervous system	- Sensory perception - Memory process - Emotional behavior

The opioid receptors have seven transmembrane helices, three extra cellular loops and three intra cellular loops as shown in Figure 2.4. These receptors have 60% homology where their transmembrane helices are highly similar. They also have a common binding cavity bearing transmembrane helices 3,4,5,6 and 7. On the other hand, their N and C termini and extracellular loops are highly divergent. The divergency of extacellular loops are related to their functions since they participate in ligand selectivity. By this way, ligands are able to bind the receptor selectively.[43]

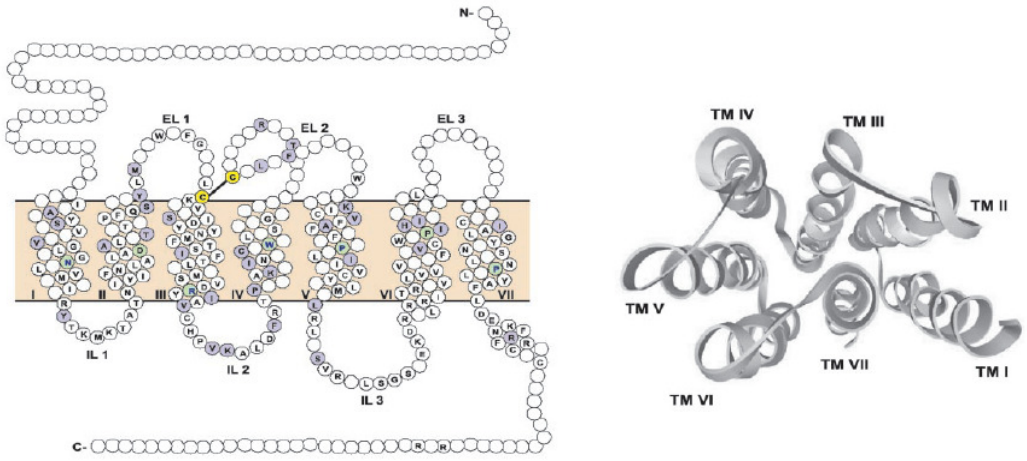


Figure 2.4 Structure of opioid receptors. EL: Extra-cellular lool, IL: Intra cellular loop and TM: transmembrane helix. [43]

The activation of opioid receptors are achieved by both endogeneous opioid peptides and alkaloid opiates. These ligands can be both selective (Table 2.2) and non selective (Table 2.3). Ligands are classified as agonist, partial agonist and antagonist. An agonist is a drug that binds to the receptor and initiate cellular response by mimicking endogeneous ligands. On the other hand, an antagonist binds to the receptor as an agonist but does not trigger any biological activity and prevent the response mediated by an agonist by occupying the receptor. A partial agonist can activate the receptor but it competes

withan agonist, hence decrease the level of the response. In the drug addiction, μ receptor plays a crucial role since its signalling is regulated by opioid agonists such as morphine [44].

Table 2.2 Selective Opioid receptor ligands [43]

Receptor	Endogenous peptides	Peptide agonists	Peptide antagonists	Agonists	Antagonists
MOP-R	Endomorphin-1 Endomorphin-2 β-endorphin β-neoendorphin Dermorphin	DAMGO PL 017	CTOP Octreotide (SMS201,995)	Fentanyl Morphine Sufentanyl	β-FNA (affinity label) Naloxonazine (irreversible)
DOP-R	Leu^5-Enkephalin Met^5-Enkephalin Met^5-Enkephalin-Arg^6-Phe^7 Met^5-Enkephalin-$Arg^6Gly^7Leu^8$ Deltorphin Deltorphin I Deltorphin II	DADLE DPDPE DSLET	ICI 174,864 (inverse agonist) TIPP TIPP[ψ]	BW373U86 SIOM SNC 80 TAN-67	Benzylidenenaltrexone (BNTX) Naltriben (NTB) Naltrindole (NTI) NTI 5′ isothiocyanate (NTII)
KOP-R	Dynorphin A Dynorphin B	Dynorphin 1a		Bremazocine Ethylketocyclazocine (EKC) Ketocyclazocine CI-977 U-50,488 Spiradoline (U-62,066) U-69,593 ICI 199,441 ICI 197,067 BRL 52,537 BRL 52,656 6′-GNTI	DIPPA Nor-binaltorphimine (nor BNI) 5′-Guanidinonaltrindole (5′-GNTI)
NOP-R[a]	Nociceptin/orphanin FQ	[Arg^{14}, Lys^{15}]nociceptin [(pX)Phe^4]nociceptin (1-13) amide analogues $NC(1-13)NH_2$ Cyclo[Cys^{10}, Cys^{14}]$NC(1-14)NH_2$ ZP120	[N-Phe^1]$NC(1-13)NH_2$ UFP-101	Ro 64-6198	Benzimidazolinone (J-113397) JTC-801 TRK-820

Table 2.3 Non selective opioid receptor ligands [43]

Receptor	Mixed agonists-antagonists	Agonists	Antagonists
MOP-R/DOP-R/KOP-R	Bremazocine	Etorphine	Diprenorphine
	Buprenorphine	Levorphanol	Naloxone
	Nalbuphine	Meperidine	Naltrexone
	Nalorphine	Methadone	β-CNA (affinity label)
MOP-R/DOP-R	DIPP-NH2Ψ		

Chapter 3

Materials and Method

3.1 Cloning of Human Kappa Opioid Receptor

3.1.1 Polymerase Chain Reaction (PCR) and TA Cloning

The cDNA of human kappa opioid receptor (*hKor*) [45] was PCR amplified. The restriction site, PstI or EcoRV/NotI and hexa-histidine tag (6xHis) were introduced using following primers at Table 3.1. PCR reaction was performed in a total volume of 50 µl containing 100 ng of the plasmid, 30 pmol of each primer, 0.2 mM dNTPs, and 2.5 unit Taq DNA polymerase (MBI Fermentas). Conditions for the 30 cycles of amplification reaction were 95 ºC for 30s, 55 ºC for 30 s and 72 ºC for 2 min. Before the first cycle reaction mixtures were kept at 95 ºC for 4 min and at the end of the 30^{th} cycle an additional 72 ºC extension period was applied for 10 min. The PCR products were subjected to agarose gel electrophoresis. The amplified products were run on 1% agarose gel at 120V for 15min.

Table 3.1 Oligonucleotide primers used for amplification of the cDNA of *hKor*. F, forward; R, antisense. Restriction sites are in bold and hexa histidine tag is underlined.

Primer Sequence
pMAL-F: 5'-AGAGTCGAC**CTGCAG**ATGGACTCCCCGATCCAGATCTTC-3'
pMAL -R: 5'-CAAGCTTGCCTGCAGCTAGTGGTGGTGGTGGTGGTGTACTGGTTTATTCATCCC-3'
pcDNA4 -F: 5'-TTCTGCA**GATATC**CAATGGACTCCCCGATCCA -3'
pcDNA4 -R: 5'-AGACTCGA**GCGGCCGC**CATACTGGTTTATTCATCCCATC -3'

In order to clone amplified PCR products, TA cloning was first performed. The 3'-A overhangs of PCR products were hybridized with 3'-T overhangs of the linearized pGEM-T Easy vector (Figure 3.1) by T4 DNA ligase. The ligation reaction was performed in a total volume of 10 μl containing 50 ng of pGEM-T Easy (Promega), 100ng of PCR product, 1x T4 DNA ligase buffer (MBI Fermentas) and 1 unit T4 DNA ligase (MBI Fermentas) for 1h at room temperature. Then, the ligation product was transformed to *E.coli DH5α* by heat shock. The transformed cells were plated on LB agar plate complemented with 100ug/ml ampiciline, 10ul of 0.1M IPTG and 40ul of 20mg/ml X-Gal and incubated at 37^0C for 16h. The following day, single white colonies were picked up and inoculated in LB liquid medium supplemented with 100ug/ml ampiciline at 37^0C for 16h to perform minipreparation of plasmid DNA (miniprep).

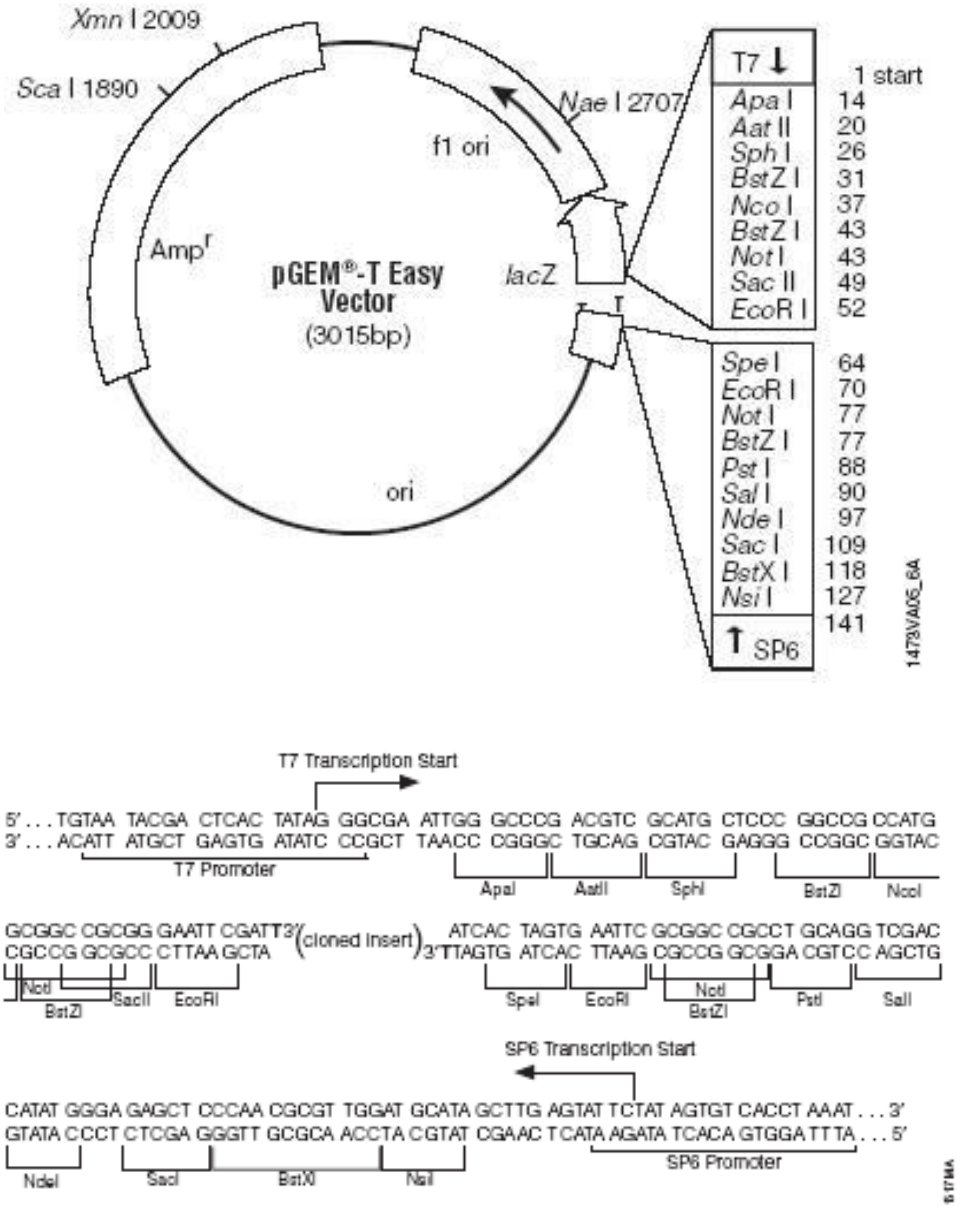
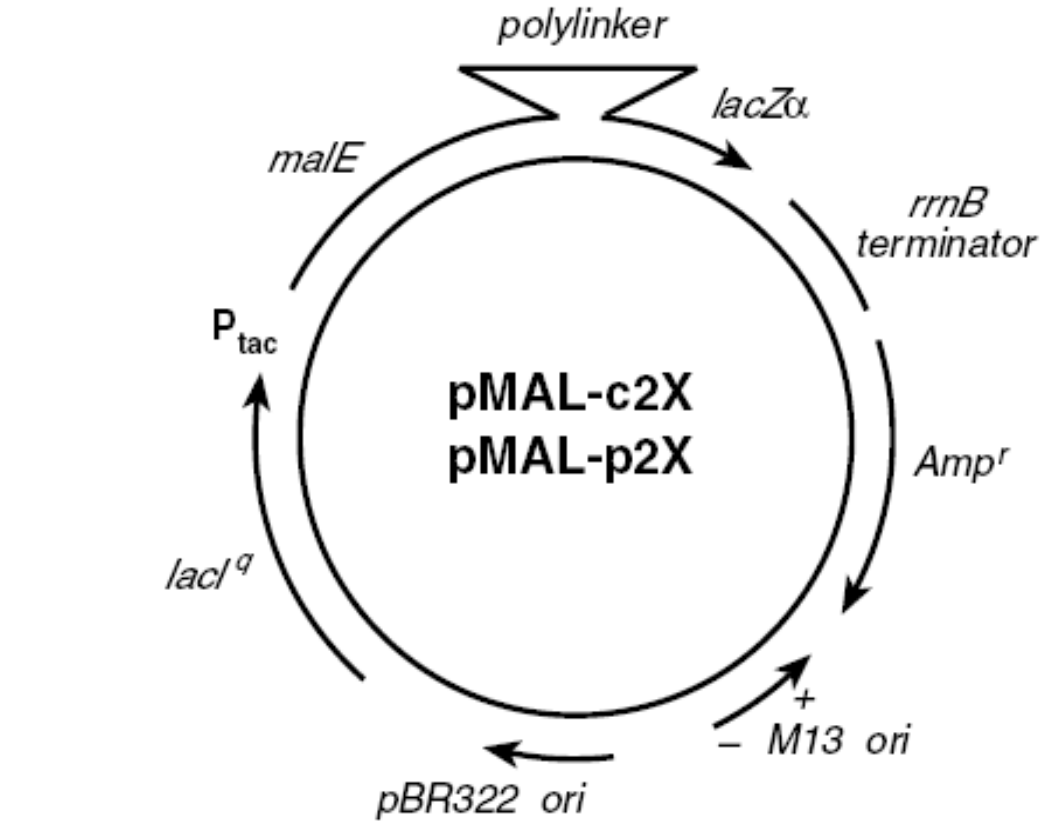


Figure 3.1. pGEM-T Easy vector map and multiple cloning sequence.

After miniprep, the presence and size of the insert was checked by restriction digestion. The plasmids were digested with EcoRI. The digestion was performed in a total volume of 20 µl containing, 300ng of plasmid DNA, 1x EcoRI buffer (MBI Fermentas) and 0.2 unit of EcoRI (MBI Fermentas) for 1h at 37^0C. The restriction digest analysis was performed with the agarose gel electrophoresis. The digested samples were run on 1%agarose gel at 120V for 15 min.

3.1.2 Cloning of *hKor* to pMAL-c2x and pcDNA4/myc-His A

In order to express *hKor* in both *E.coli* DH5α and HEK293T, *hKor* was cloned to bacterial expression vector, pMAL-c2x (Figure 3.2) and mammalian expression vector, pcDNA4/myc-His A (Figure 3.3), respectively. First, the pMAL-c2x and pcDNA4/myc-His A vectors were digested with PstI and EcoRV/NotI respectively. Then, *pGEM-T Easy – hKor* plasmid was digested with the same restriction enzymes. The digestion was performed in a total volume of 100 µl containing, 1ug of plasmid DNA, 1x Buffer O and 1 unit of PstI (MBI Fermentas) or 1 unit of NotI (MBI Fermentas) / 2 units of EcoRV (MBI Fermentas) for overnight at 37^0C. The digested samples were run on 1% agarose gel at 90V for 2-3 hrs. The samples were gel-purified with the gel purification kit (Qiagen). The ligation was performed as in section 3.1.2 with 1:3 molar ratio of vector:insert. The newly formed plasmids were transformed to *E.coli* DH5α by heat shock and seeded on the LB agar plate with 100ug/ml ampiciline. After selection of single colonies in LB/ampiciline liquid medium, miniprep was performed. The presence of the insert was checked with restriction digestion as in section 3.1.1. The digestion was performed in a total volume of 20 µl containing, 300ng of plasmid DNA, 1x Buffer O (MBI Fermentas) and 0.2 unit of PstI or 0.2 unit of EcoRV/0.4 unit of NotI (MBI Fermentas) for 1h at 37^0C.



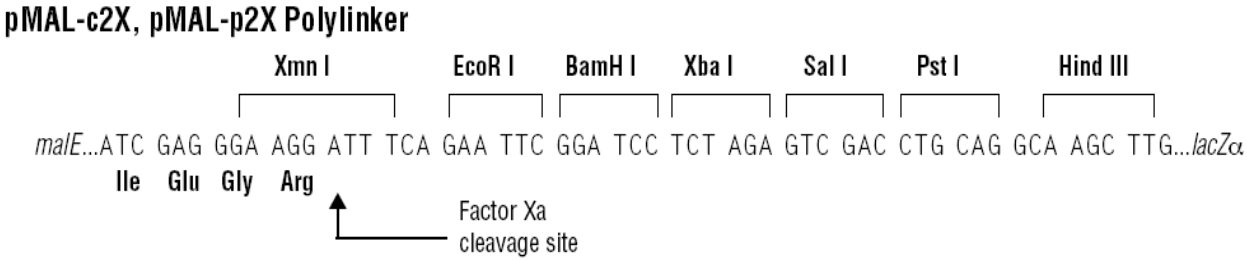
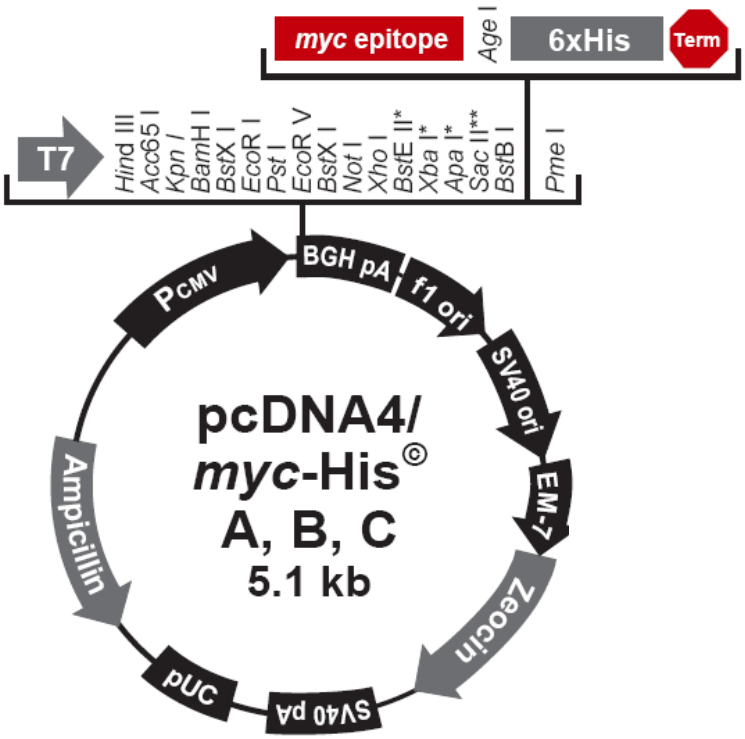


Figure 3.2. pMAL-c2x vector map and multiple cloning sequence.



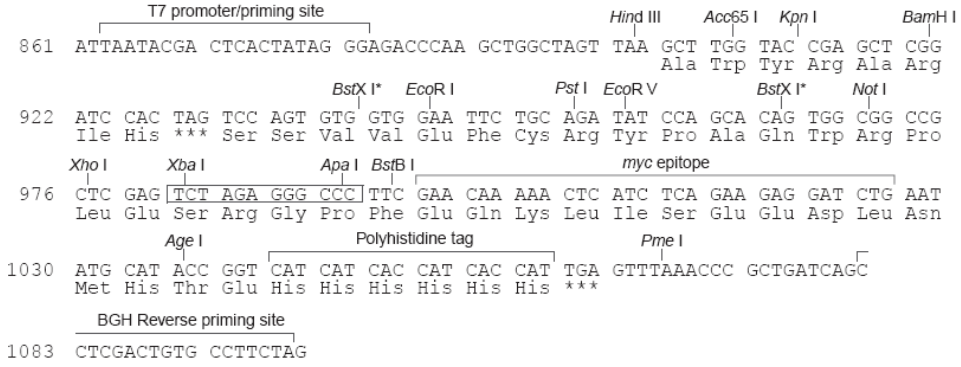


Figure 3.3. pcDNA4/myc-His A vector map and multiple cloning sequence.

3.2 Expression and Purification of Human Kappa Opioid Receptor

The single colony possessing *pMAL-c2x-hKor* plasmid was picked up and inoculated in LB liquid medium supplemented with 100ug/ml ampiciline at 37C for 16h Then, overnight culture was diluted 1:100 and inoculated in LB/ampiciline liquid medium. Protein expression was induced at OD_{600}=0.6 with 0.5mM IPTG for 3 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.

3.3 SDS-PAGE and Western Blotting

SDS–PAGE was performed using a Bio-Rad Mini-PROTEAN III electrophoresis cell. Protein samples were electrophoresed on a 10% separating gel. Gels were run at 150 V for 1.5 h. After SDS–PAGE, gels were transferred to polyvinylidene difluoride membrane (Biotrace PVDF, Pall Corporation, FL, USA) with a Mini-Trans-Blot electrophoretic

transfer cell (Bio-Rad) at 90V for 1 hr. After pre-blocking with 5% BSA dissolved in Tris-buffered saline (TBS), the membrane was incubated with anti-His (1:250 diluted in 0.05% Tween20/TBS) (Santa Cruz) for 1 hr at room temperature. After a series of washes the membrane was subsequently incubated with AP-conjugated secondary anti-mouse IgG antibody (1:10000 diluted in 0.05% Tween20/TBS) (S41176, Sigma) for 1 hr. Proteins were visualized by NBT/BCIP detection system (Roche).

3.4 Immobilization of Human Kappa Opioid Receptor

Immobilization of purified hKOR to the gold surface was achieved by Dithiobis (succinimidyl propionate) (DSP) and Nα,Nα -bis(carboxymethyl)-L-*lysine* (*NTA*) (Lys-NTA) complex as in [11]. First, 10mM of Lys-NTA (Fluka) and 1mM DSP (Fluka) were reacted in 100mM $NaHCO_3$ for 3h at room temperature. The gold surface was then subjected to newly formed DSP-Lys-NTA complex for 2h at room temperature. After the surface was washed several times with distilled water, the surface was treated with 50mM of $NiCl_2$. The protein (100ug/ml) was allowed to be immobilized on gold surface, which was equilibrated previously with the solubilization buffer without β-mercaptoethanol, for 1hr with gentle shaking. Figure 3.4 demonstrates the schematics of the surface functionalization of hKOR on the gold surface of the cantilever array.

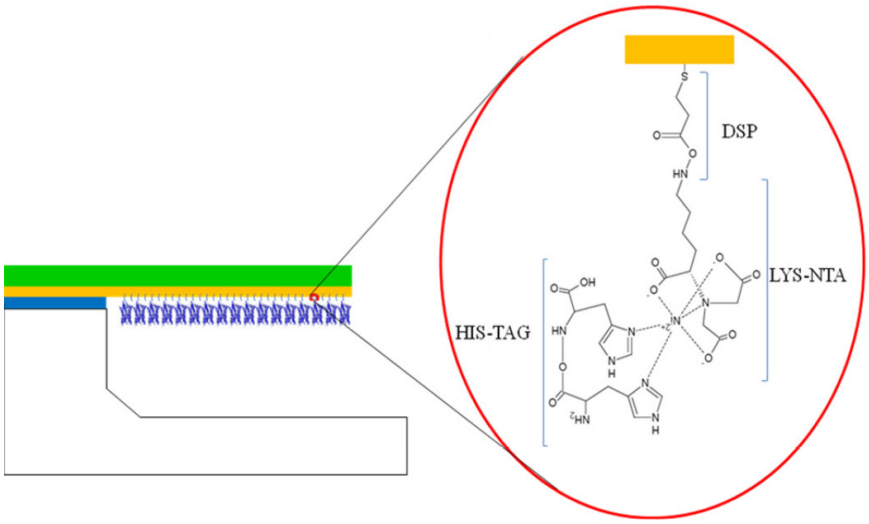


Figure 3.4 Schematics of the surface functionaliztion of hKOR.

3.5 Fluorescence Microscopy

The immobilization of the protein was verified with fluorescence microscopy. The schematics of the detection of the surface functionalization was exhibited in Figure 3.5. The 25mm^2 gold surface was functionalized as in section 3.4. Then, the surface was treated with 5% BSA (diluted in 0.05% Tween20/TBS) to prevent non-specific binding of antibodies. After it was washed 3 times with 0.05% Tween20/TBS, the surface was subjected to anti-His (1:250 diluted in 0.05% Tween20/TBS) (Santa Cruz) for 1h at room temperature and washing was performed as above. The surface was treated with FITC-conjugated anti mouse IgG (1:250 diluted in 0.05% Tween20/TBS) (Promega) for 1hr. Also, the same procedure was repeated with another gold surface where the protein was not introduced to see whether antibodies bind non-specifically to the surface.

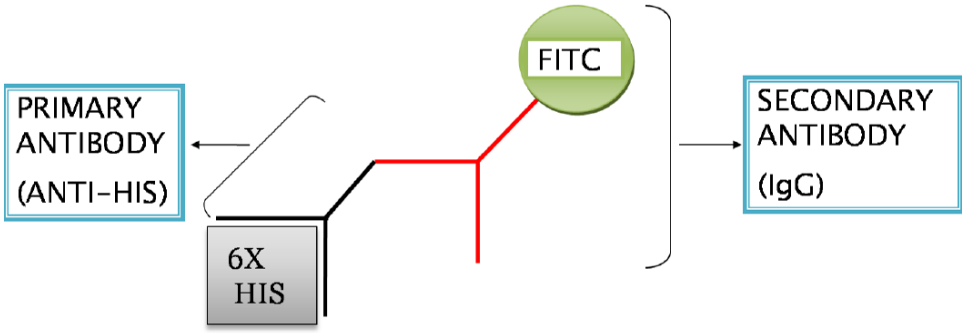


Figure 3.5 Schematics of the detection of immobilized hKOR.

3.6 AFM

The immobilization and conformation of the protein on the surface was observed with AFM. The 25mm^2 gold surface was functionalized as in section 3.4. As a reference, another gold surface was functionalized only with DSP-Lys-NTA complex.

3.7 Bio-sensitivity and Bio-selectivity of Cantilever Arrays

To conduct bio-sensitivity and bio-selectivity measurements of cantilever arrays, the surface functionalization of chips possessing gold coated cantilever arrays was performed as at Table 3.2. For bio-sensitivity measurements, two chips were used: The reference chip and the sample (hKOR) chip. The hKOR was immobilized on the chip as in section 3.5. The reference chip was only subjected to the solubilization buffer without β-mercaptoethanol. Then, the sample chip was treated with anti-His (1:250 diluted in 0.05% Tween20/TBS) (Santa Cruz) for 1h at room temperature and rinsed 3 times with 0.05% Tween20/TBS and then with water. For bio-selectivity measurements, three chips were used: The reference chip, the sample (hKOR) chip and the control (BSA) chip. The hKOR was immobilized on the chip as in section 3.4. Immobilization of BSA was achieved by

DSP crosslinker. The cantilever arrays on the control chip were subjected to 1mM of DSP for 30min at room temperature. The chip was first rinsed with DMSO and then with water. One mg/ml BSA dissolved in Phosphate-buffered saline (PBS) was immediately added to activated surface of the chip. After BSA was incubated for 1-2hr at room temperature, the chip was rinsed with PBS to remove cross-linker by-products and unconjugated protein. Following the functionalization of the sample chip and the control chip, the chips were subjected to anti-His (1:250 diluted in 0.05% Tween20/TBS) for 1h at room temperature and washed 3 times with 0.05% Tween20/TBS and then with water. The reference chip was treated with 0.05% Tween20/PBS, 0.05% Tween20/TBS and water. Figure 3.6 demonstrates the schematics of the bio-selectivity experiment.

Table 3.2 Summary of the process for bio-sensitivity and bio-selectivity

			Bio-selectivity	
			Bio-sensitivity	
Step #	**Process**	**Chip: Reference**	**Chip: hKOR**	**Chip: BSA**
0	Surface cleaning	✓	✓	✓
1	Protein Binding	x	hKOR	BSA
2	Washing	✓	✓	✓
3	Antibody Binding	x	✓	✓
4	Washing	✓	✓	✓

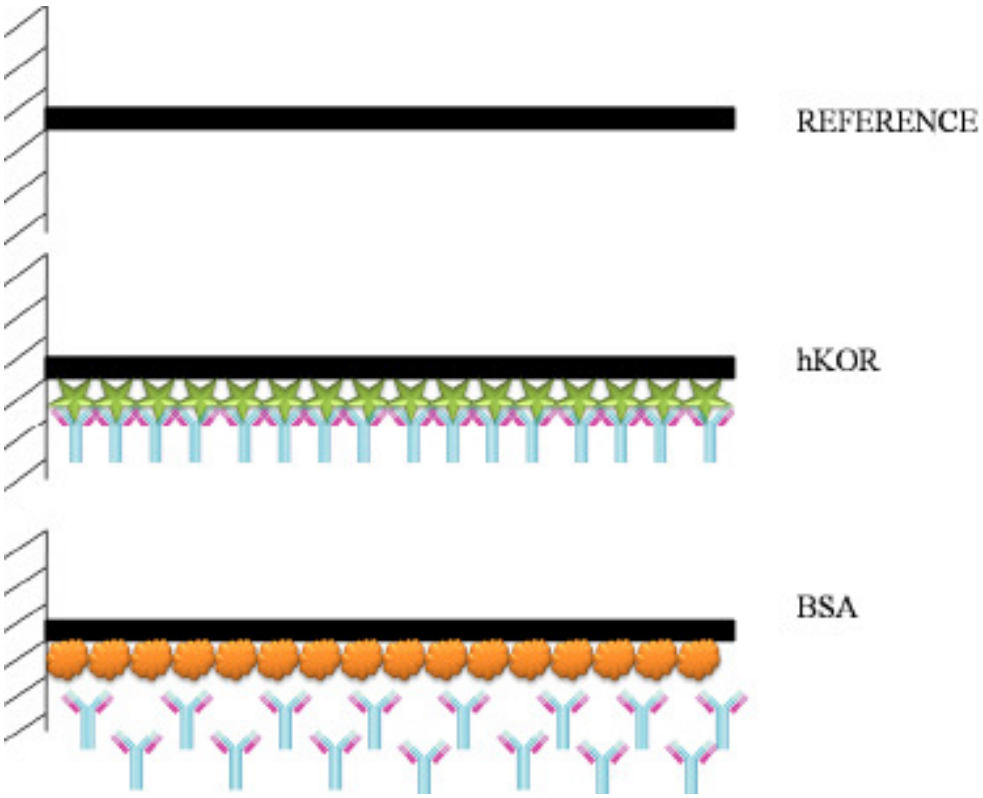


Figure 3.6 Schematics of bio-selectivity experiment. hKOR is in green, BSA is in orange and anti-His is in blue.

Chapter 4

Results and Discussion

4.1 Cloning of Human Kappa Opioid Receptor

The cDNA of hKor was amplified with PCR. The amplified products were analyzed with agarose gel electrophoresis. Figure 4.1 shows agarose gel analysis of PCR products. The PCR products are at expected size, 1143bp and there is not any non-specific amplification. Since the products have a single band, they were used for TA cloning directly.

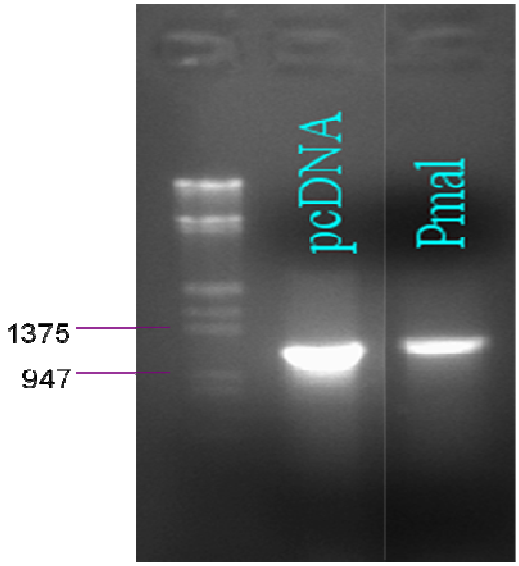


Figure 4.1 Agarose gel analysis of PCR products. Lane 1: Marker, lane 2: Amplified *hKor* for pcDNA4/myc-His A and lane 3: Amplified *hKor* for pMAL-c2x

After TA cloning was performed, the newly formed *pGEM-T Easy-hKor* plasmid was digested with appropriate restriction enzymes. The Figure 4.2 shows the agarose gel analysis of digested samples. The linear vector is at 3010bp and *hKor* is at 1143bp. Then, *hKor* was gel purified to be cloned to pcDNA4/myc-His A and pMAL-c2x.

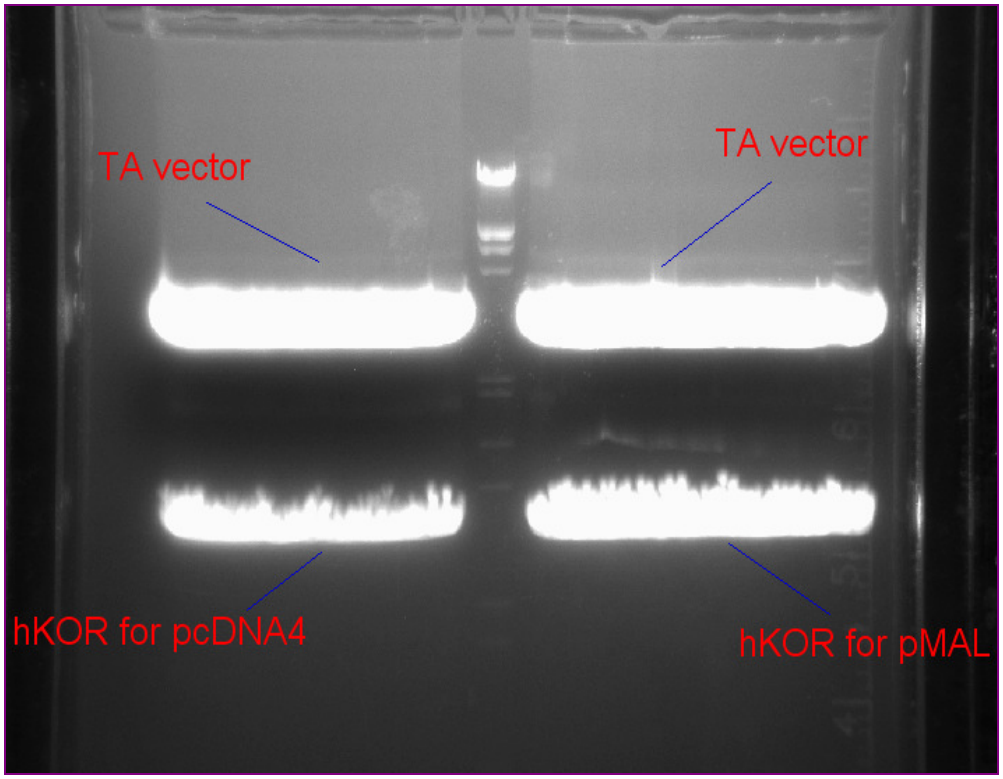


Figure 4.2 Agarose gel analysis of restriction digestion. Lane 1: hKor for pcDNA4/myc-His A in pGEM-T Easy, lane 2: Marker and lane 3: hKor for pMAL-c2x in pGEM-T Easy

In order to express *hKor* in both *E.coli* DH5α and HEK293T, *hKor* was cloned to bacterial expression vector, pMAL-c2x and mammalian expression vector, pcDNA4/myc-His A, respectively. Figure 4.3 shows the restriction analysis of newly formed plasmids possessing the insert (*hKor*).

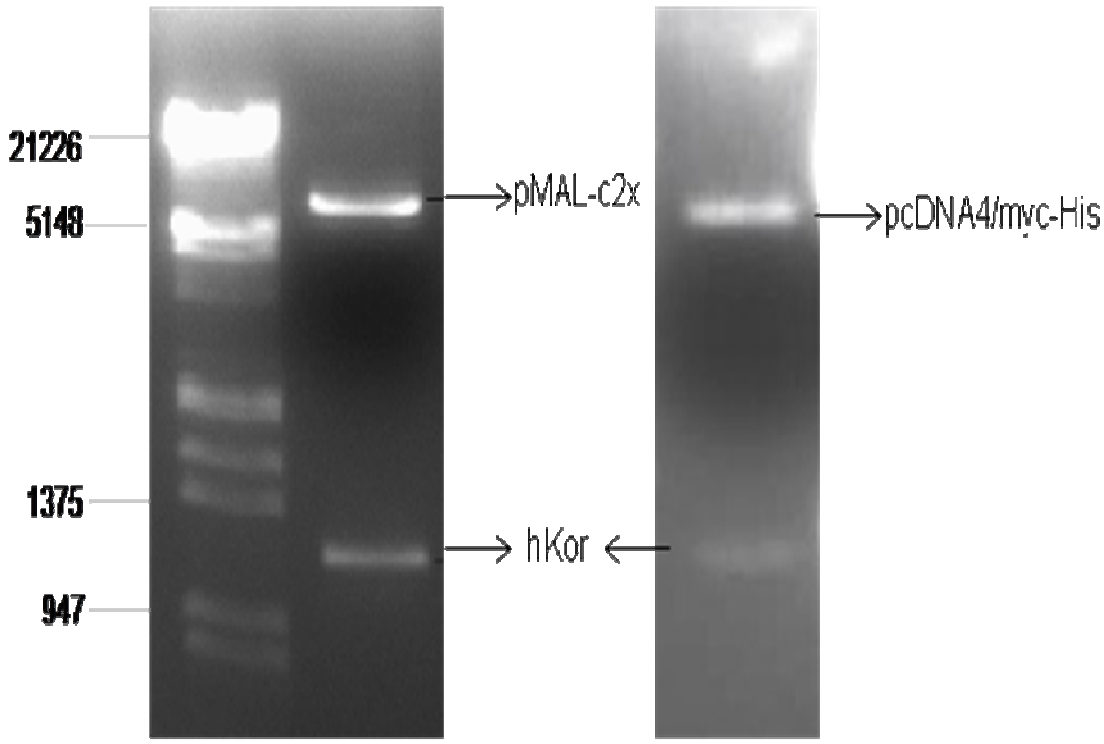


Figure 4.3. Digestion of *pMAL-c2x-hKor* and *pcDNA4/myc His A - hKor*

4.2 Expression and Purification of Human Kappa Opioid Receptor

The cDNA of *hKor* was cloned to downstream of *malE* gene, which encodes maltose binding protein (MBP), in the pMAL-c2x. The expression of HKOR was induced in *E.coli* DH5α at 37^0C and monitored by the Western Blot analysis (Figure 4.4, lane 3). The apparent molecular weight of hKOR is 85kDa with fusion protein MBP (~40kDa). The expression level of hKOR is low in *E.coli* (lane 3) . It was previously reported that the overexpression of higher eukaryotic membrane proteins in bacteria yields low expression levels [46]. To increase expression levels, soluble, highly expressed proteins were fused to these proteins. In a recent study, functional human delta, kappa and mu opioid receptors were expressed in E.coli as fusions to MBP [47]. The reported yields of opioid receptors are also low. It is stated that the main factors limiting the yield of receptors may be present at the level of translation, membrane insertation and folding. Rare codons in cDNA of receptors could cause frameshift and deteriorate the translation [48]. Moreover, the overexpression of these proteins was toxic and cause plasmid loss after induction. Although it is revealed as the decrease in the growth temperature of bacteria increase the expression of the protein by enhancing proper folding and altering membrane chemistry [47], an increase at the expression level at given conditions could not be detected in the present study. In fact, only MBP and/or truncated forms of the protein were observed.

Beside the expression level, the functionality of the recombinant receptors is also important for ligand binding assays. It is clearly indicated that MBP fusion protein does not affect the ligand binding and the recombinant receptors have the same activity with native receptors expressed in COS cells [47]. However, higher growth temperatures yields lower functional proteins [47]. Since the expression was optimized at 37^0C, the functionality of recombinant receptors should be further investigated for this study. Moreover, hKOR can be expressed in HEK293T to obtain functional native receptors.

Since the present study does not address the functionality of the receptors, the expression of the recombinant receptor at given conditions served well to the aim of the study.

After the recombinant hKOR was successfully expressed, the purification was performed by Ni-NTA resin. The 6xHis extension at the C-terminus of the receptor enables one-step purification. The Figure 4.4 shows the western blot analysis of the purification of hKOR (lane 4-10). The recombinant receptors were first solubilized with SDS. The choice and concentration of the detergent is a crucial step to have high solubilization efficiency and maintain the functionality of the receptor. According to solubilization analysis of human mu receptor expressed in *Pichia pastoris*, 0.1% SDS has100% solubilization efficiency [49]. However, 1% DM was used for conserving the functionality of several GPCRs [50-52] despite its low solubilization efficiency [49]. Since the functionality of the receptor is not the main concern of this study, 0.1% SDS was used to have high solubilization efficiency. Although 100% solubilization efficiency was not observed in *E.coli*, the solubilization of hKOR was mostly achieved since the protein concentration is low at solubilized pellet (Figure 4.4, lane 5). After the receptor was successfully solubilized, the Ni-NTA batch purification was performed. The solubilized receptor was purified efficiently (lane 10) although it remained partially bound to resin (lane 9). Furthermore, the receptor remains intact and stable under this purification system since there was not any degradation product observed.

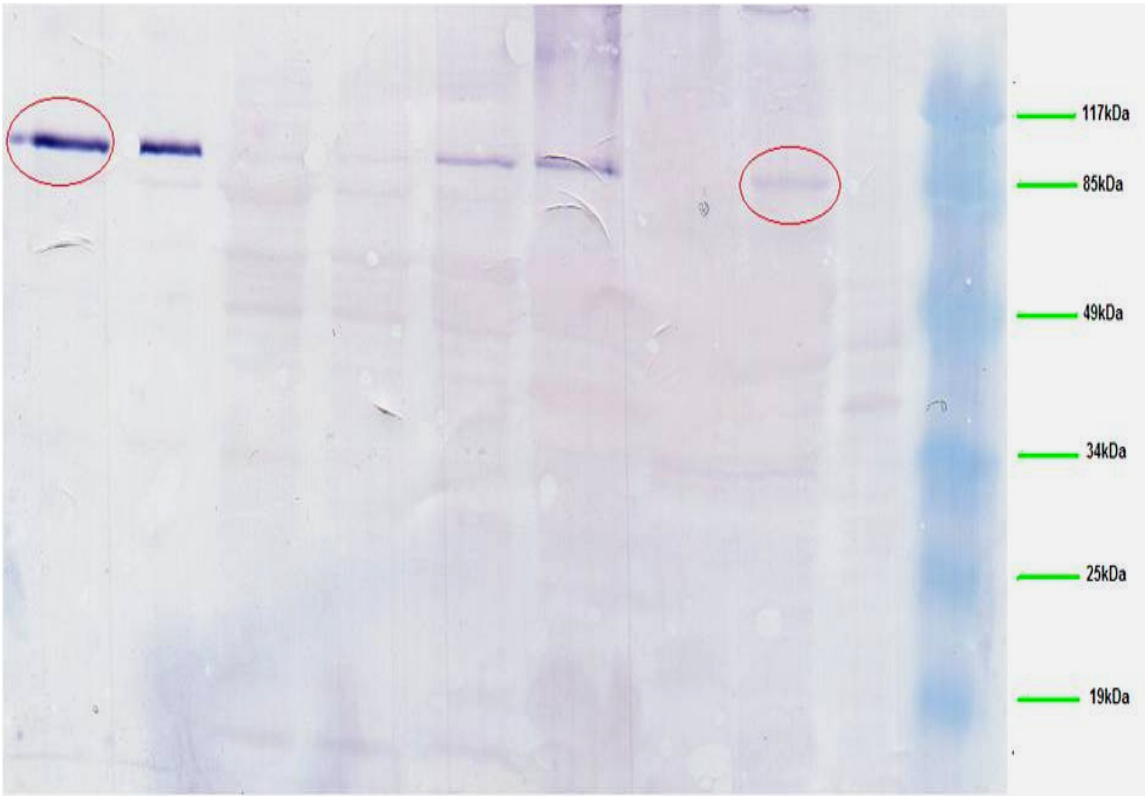


Figure 4.4 Western blot analysis of expression and purification of hKOR. (From right to left) lane 1: Molecular Weight Marker, lane 2:Before IPTG induction, lane 3: After IPTG induction, lane 4: Cell free extract, lane 5: Solubilized pellet, lane 6: Flow-through, lane 7: Wash 1 lane:8: Wash 2, lane 9: Resin and lane 10:Elute

4.3 Immobilization of Human Kappa Opioid Receptor

Immobilization of purified hKOR to the gold surface was achieved by DSP and Lys-NTA complex as in [11]. DSP crosslinker contains both a thiol group and an amine-reactive *N*-hydroxysuccinimide (NHS) ester. The thiol group enables DSP to be immobilized on the gold surface via thiol-gold interaction whereas NHS ester reacts with Lysine of the Lys-NTA, which provides immobilization of the protein. The immobilized DSP-Lys-NTA complex captures Ni^{2+} and forms a coordination compound with 6xHis tag of hKOR. The immobilization of the protein was verified with fluorescence microscopy. The anti-His antibody recognizes the 6xHis tag of hKOR. When FITC-conjugated anti-mouse IgG is introduced, the secondary antibody recognizes the primary antibody and the protein becomes detectable under fluorescence microscope via FITC.

Immobilization of proteins on the gold surface was observed using fluorescence microscopy as shown in Figure 4.5 where green spots represent immobilized protein molecules. No signal is observed at the blank surface (Figure 4.5B), which indicates that antibodies do not bind non-specifically and signals observed at Figure 4.5A are possessed by immobilized protein molecules.

AFM study was also carried out to observe conformation of immobilized proteins. Figure 4.6A demonstrates crosslinker functionalized surface. The crosslinker is distributed evenly and formed SAM. In Figure 4.6B, the surface was functionalized with hKOR. However, the distribution of the protein was uneven and in the form of aggregates. Moreover, the immobilization of the protein was poorly achieved since the surface distribution of proteins is low. Nevertheless, the immobilization and conformation of proteins are observed.

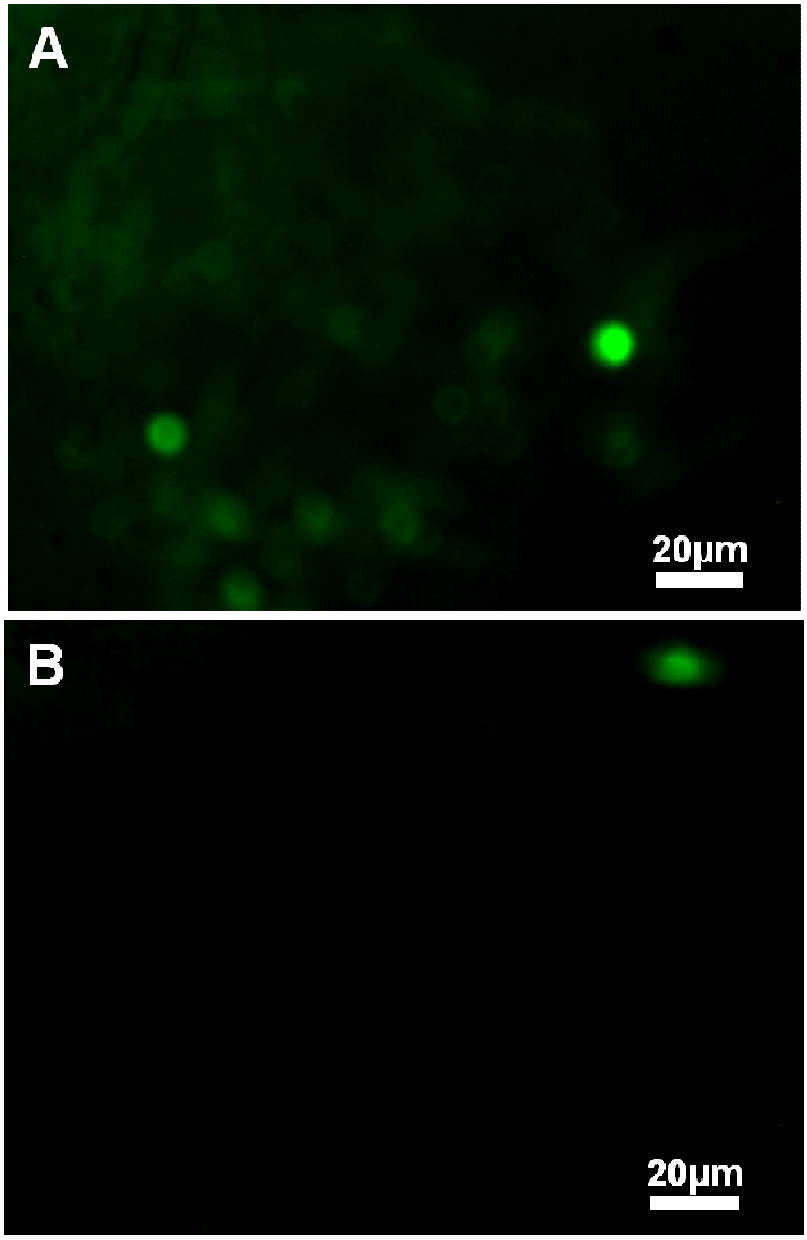
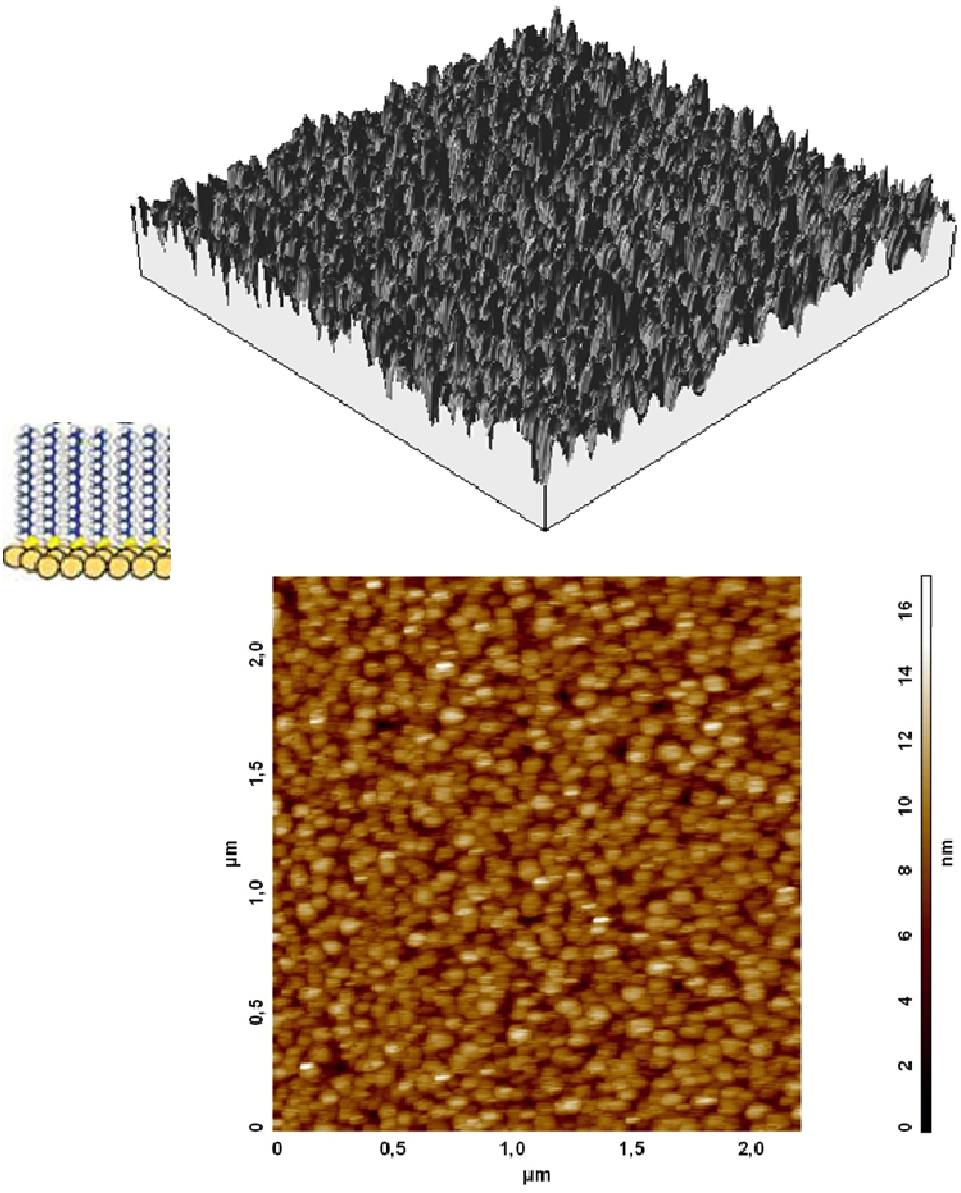


Figure 4.5 Fluorescent micrographs of immobilized proteins. A. Immobilized hKOR on gold surface treated with FITC conjugated IgG (1:250) B. Blank gold surface treated with FITC conjugated IgG (1:250).

A



B

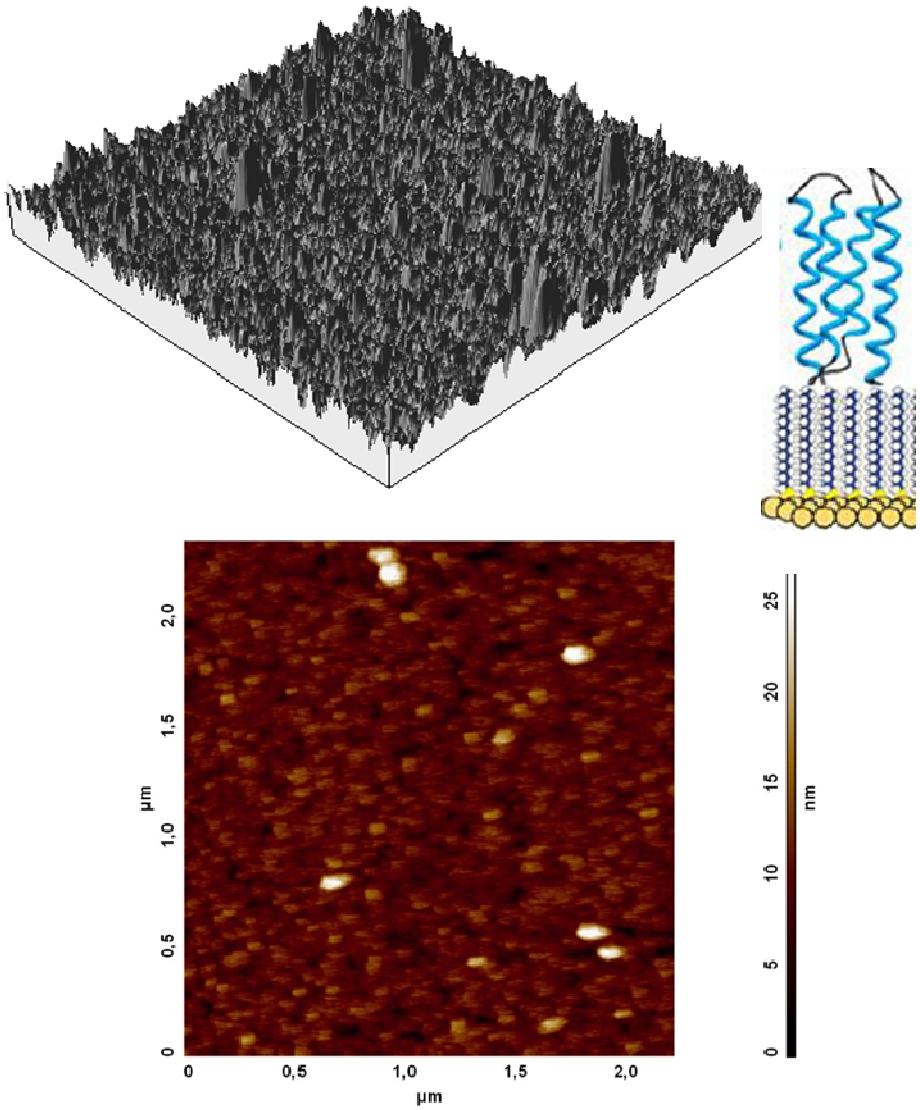


Figure 4.6 AFM images of immobilized hKOR. A. Crosslinker functionalized surface B. hKOR functionalized surface.

4.4 Bio-Sensitivity and Bio-Selectivity of Cantilever Arrays

After the immobilization of proteins was achieved, the ability of cantilever arrays to detect and select the target bio-molecule was tested by mass measurements. 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 this approach a diffraction grating was integrated to the tip of each cantilever for interferometric measurements. Using a photo diode setup the resonance frequency could be picked up with good accuracy.

4.4.1 Bio-Sensitivity

The results of mass measurements prior and after the functionalization of cantilever with a length of 70 μm are given in Figure 4.7. The total shift in the frequency amounts to 180 Hz which corresponds to a mass accumulation of 85 pg. The corresponding mass accumulation is higher than expected. Since hKOR is a membrane protein, it is not clear if it forms SAMs, double layer or aggregates as micelles. Yet, the environmental effects as humidity and temperature on measurements and contaminations emerged from each process are not clarified. Nevertheless, the cantilever arrays can sense the immobilized proteins.

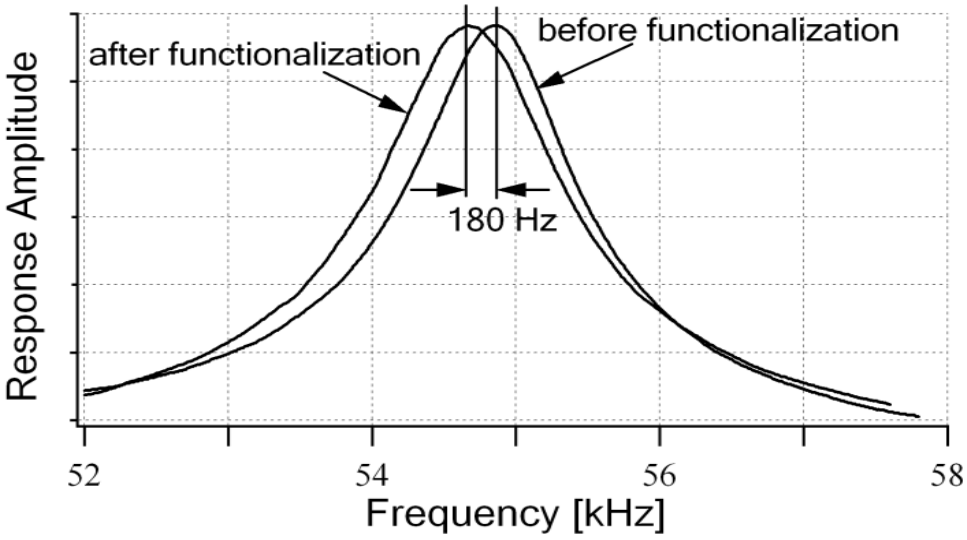


Figure 4.7 The shift in resonance frequency of a 7x70 μm cantilever upon exposure to opioid receptors.

Since it was verified that the cantilever arrays are able to detect the immobilized proteins, the bio-sensitivity of the arrays was tested with using an antibody that recognizes the immobilized proteins, anti-His. The measured resonance frequencies are shown at Table 4.1. The measurements for reference chip and functionalized chip were taken after fabrication, functionalization and antibody binding (refer to chapter 3, section 3.7). After functionalization, the frequency shift at the reference chip is unexpectedly high. This shift hints the effect of solution, which contains 50% glycerol. Hence it is determined that glycerol was accumulated on the chip. However, the accumulated mass shows high deviation over cantilevers having similar resonance frequencies. Then, it is concluded that glycerol distribution is uneven over the chip. Although the accumulated mass is unexpectedly high and its distribution is uneven, it is clearly seen that the accumulated mass at the functionalized chip higher at each cantilever.

Table 4.1 The resonance frequency shift in reference and functionalized chips.

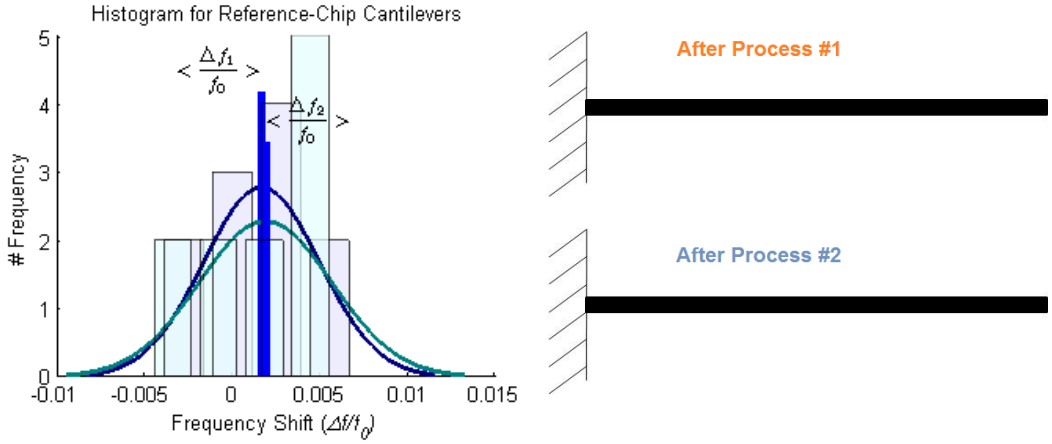
	Initial (kHz)	Functionalization (1) (kHz)	Antibody binding (2) (kHz)	Resonant frequency shift (1) (kHz)	Resonant frequency shift (2) (Hz)	Mass accumulation (1) (pg)	Mass accumulation (2) (pg)
Reference Chip	121.25	120.20	120.46	-1.05	260	86.274	-21.201
	124.05	121.75	122.01	-2.30	260	187.518	-20.932
	98.70	94.47	94.57	-4.23	100	445.263	-10.279
	113.90	109.08	109.05	-4.82	-30	439.285	2.676
Functionalized Chip	193.93	189.07	188.51	-4.86	-560	347.842	39.749
	216.36	208.55	207.77	-7.81	-780	509.707	50.251
	188.60	181.95	181.66	-6.65	-290	497.230	21.346
	186.81	179.16	178.51	-7.65	-650	582.663	48.738
	191.65	185.22	184.56	-6.43	-660	471.867	47.864

Since the functionalized chip was treated with the same solution, glycerol in the solution is also responsible for the extra mass accumulation. However, the mass accumulation at the functionalized chip is higher compared to reference chip, thus, it is concluded that the surface functionalization was achieved. After antibody treatment, mass loss is observed for the reference chip. This loss indicates that glycerol was removed during the washing step. Moreover, the mass accumulation at the functionalized chip is due to antibody binding, since the measured mass is consistent with the expected mass of the antibody and there is not any mass accumulation at the reference chip.

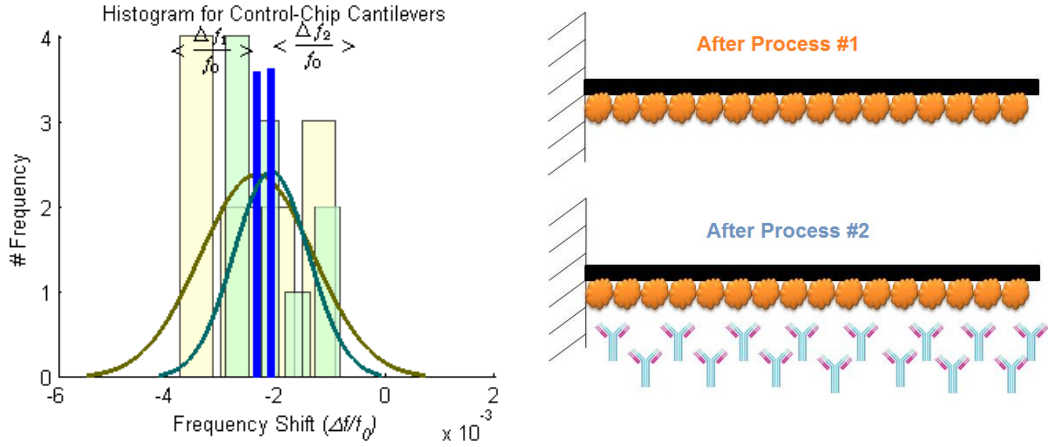
4.4.2 Bio-Selectivity

It was verified that cantilever arrays were able to sense bio-molecules, however their ability to select only the target bio-molecule was unclear. To test whether the cantilever arrays are selective to a specific bio-molecule, an additional chip was functionalized with a non-interacting protein, BSA, as the control. Figure 4.8 exhibits average shifts in resonance frequencies of cantilevers on reference, hKOR and BSA functionalized chips after functionalization and antibody treatment. It is evident that hKOR and BSA were successfully functionalized on the chips since there is a negative shift at both kKOR and BSA chip (Figure 4.8B and C respectively), but no negative shift is observed at reference chip (Figure 4.8A). Moreover, hKOR and BSA have similar molecular weights as 85kDa and 69kDa respectively, hence the expected shift should be compatible at each chip. It is clearly seen that the shifts are similar at both hKOR and BSA functionalized chips after functionalization. It should be noted that the unexpectedly high mass accumulations are not observed in this experiment, because the solution effect was minimized with replacing glycerol solution with TBS or PBS. After the immobilization of hKOR and BSA was verified, the chips were treated with the antibody that recognizes hKOR. The shift in resonance frequencies of cantilevers on hKOR functionalized chip after antibody treatment compared to BSA functionalized chip clearly indicates that cantilevers arrays are able to sense and select the target bio-molecule since a negative shift is observed only at hKOR functionalized chip and there is no change at reference chip and an insignificantly small shift at BSA functionalized chip. It should be clearly indicated that the antibody that recognizes hKOR is twice the molecular weight of hKOR, hence the mass accumulation should be doubled after antibody binding. However, the corresponding mass accumulation on the hKOR functionalized chip is lower than expected.

A


Histogram for Reference-Chip Cantilevers
Frequency
Frequency Shift (Δf/f0)
After Process #1
After Process #2

B


Histogram for Control-Chip Cantilevers
Frequency
Frequency Shift (Δf/f0)
x 10-3
After Process #1
After Process #2

C

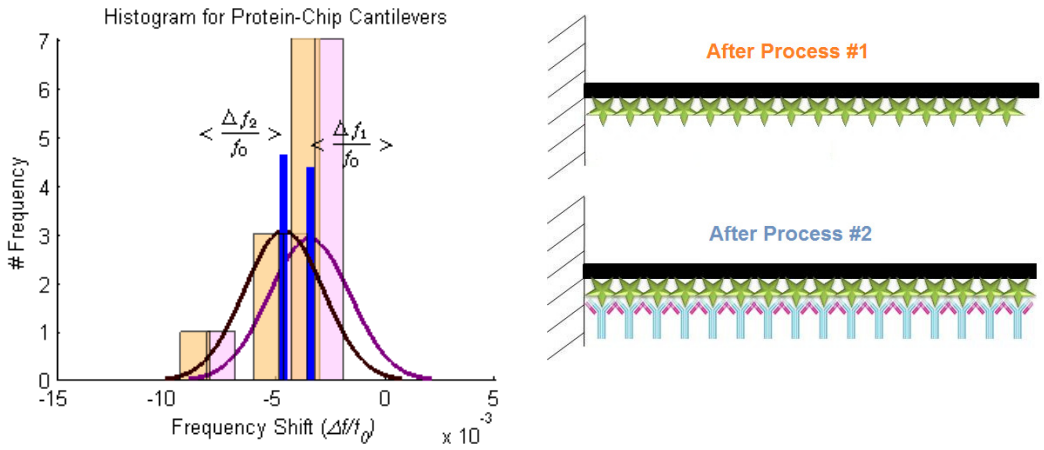


Figure 4.8 The average shift in resonance frequency of cantilevers on reference, hKOR and BSA functionalized chips after functionalization and antibody treatment.

CONCLUSION

This study indicated that human kappa opioid receptors were successfully immobilized on a cantilever array biosensor for the first time and a specific detection of interaction between hKOR and His-antibody has been demonstrated. As the extension of this work, the activity of the receptor can be studied. For this purpose, expression and purification of the receptor can be optimized to obtain active receptors, the activity of the receptor can be compared before and after functionalization, by this way, the capability of the functionalization method to conserve the activity of the receptor can be determined. Moreover, the activity of the receptors can be monitored with respect to time and temperature. Ligand binding assays can be also performed depending on the concentration of the ligand. Furthermore, ligand binding assays can be carried out with blood samples. Since this study only focuses on the kappa type receptor, other type of receptors can be immobilized to detect broad range of drugs.

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