The C-Terminus of the mu Opioid Receptor Is Critical in G-Protein Interaction as Demonstrated by a Novel Graphene Biosensor

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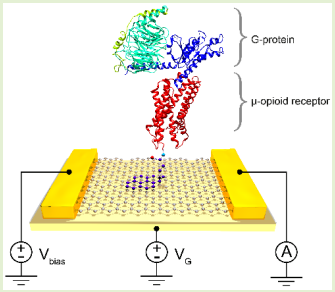
***Abstract*—Several water-soluble variants of the human mu opioid receptor (wsMORs) have been designed and expressed, which enables the detection of opioids in the nM to pM range using biosensing platforms. The tools previously developed allowed us to investigate MOR and G-protein interactions in a lipid free system to demonstrate that the lipid bilayer might not be essential for the G-protein recognition and binding. In this study, we are able to investigate G-protein interactions with MOR by using graphene enabled technology, in a lipid free system, with a high sensitivity in a real time manner. A new wsMOR with the native C-terminus was designed, expressed and then immobilized on the surfaces of scalable graphene field effect transistor (GFET)-based biosensors, enabling the recording of wsMOR/G-protein interaction with an electronic readout. G-protein only interacts with the wsMOR in the presence of the native MOR C-terminus with a K_A of 32.3±11.1 pM. The electronic readout of such interaction is highly reproducible with little variance across 50 devices in one biosensor array. For devices with receptors that do not have the native C-terminus, no significant electronic response was observed in the presence of G-protein, indicating an absence of interaction. These findings reveal that lipid environment is not essential for the G-protein interaction with MOR, however, the C-terminus of MOR is essential for G-protein recognition and high affinity binding. A system to detect MOR-G protein interaction is developed. wsMOR-G2_Cter provides a novel tool to investigate the role of C terminus in the signaling pathway.**



***Index Terms*—Biosensors, C-terminus, G-protein, graphene, mu opioid receptor, water-soluble.**

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I. Introduction

G-PROTEIN coupled receptors (GPCR) are a family of receptor proteins essential for many types of biological signal transmission and the target of a large percentage of modern drugs [1]. The mu opioid receptor (MOR) is one of the extensively studied class A GPCRs due to its critical role in the regulation of various physiological functions including pain modulation, diarrhea suppression, addiction, and respiratory depression.

We have designed and characterized several water-soluble variants of the human MOR (wsMORs) by computationally replacing lipid-facing amino acid residues located in the transmembrane (TM) domain [2], [3]. Such highly engineered receptors are soluble in water without needing a high concentration of detergents, which makes the receptors easy to handle and greatly increases the feasibility of immobilizing them on the surface of microchips or biosensors for opioid detection in the nM to pM range [4], [5]. We hypothesized that these variants may also offer an opportunity to investigate the interaction of the receptor with G-proteins (guanine

nucleotide-binding proteins) by a biosensor enabled system. It can be used to demonstrate whether the lipid system plays an essential role in G-protein interactions with MOR. Recently the structure of the murine MOR in complex with the Gi protein, mMOR–Gi-protein, was solved providing a detailed structural framework for the interaction between these two entities [6]. Despite this new knowledge, structural information regarding the detailed function of the receptor's intracellular C-terminus in influencing this interaction, and that with other intracellular proteins is absent even though its relevance is well established [7]. Moreover, resolving the dynamic interactions between MOR and G-proteins or other intracellular effectors and modulators remains elusive, especially in a manner where these interactions are separated from the contributions of lipid bilayers.

Due to its superior electron carrier mobility and biocompatible properties [8], [9], graphene field effect transistor (GFET)-based biosensor is an ideal platform for the immobilization and characterization of biomolecules [10]–[12]. Previous studies have demonstrated the use of GFETs for ultrasensitive detection of biomolecules, such as DNA [13], adenosine triphosphate (ATP) [14] and small-molecule drugs [15]. Conventional methods of detecting such interactions often need complicated equipment, professional operations, and time-consuming work process [13], [16]. The fabrication of GFETs is compatible with modern semiconductor technology and can be produced in very large scale with excellent device-to-device uniformity [10], which could greatly decrease the workload of the sensing procedure. In addition, the readouts of GFET sensors are electrically based, which can generate a rapid response and decrease the complexity of sensing equipment, benefiting the cost and speed of detection.

In this report, we demonstrate a graphene-enabled biosensor to monitor MOR–G-protein interactions, which will help to elucidate the real time dynamics of G protein interactions with the receptor. We investigated the specific role of the C-terminus in the G-protein binding. A new wsMOR with the native C-terminus (namely, wsMOR-G2_Cter) was designed, expressed, and then immobilized on the GFET surface. When exposed to the G-protein, a carrier concentration change in graphene channel was induced by G-protein interacting with wsMOR-G2_Cter, resulting in a shift of the charge neutral point, or so-called Dirac voltage. By measuring and analyzing this Dirac voltage shift, we were able to show that the G-protein can interact with wsMOR-G2_Cter specifically. This proof-of-concept experiment demonstrates the feasibility of this approach to detect G-protein interactions with MOR using an electronic readout and establishes a novel approach for measurement of G-protein interactions with GPCRs in a highly sensitive manner.

II. Materials and Methods

A. Expression and Purification of wsMOR-G2_Cter and G-Protein

The water-soluble variants of MOR are expressed and purified in an *E. coli* system as we reported previously [2], [3]. Briefly, we expressed and purified two proteins, including the computationally designed water soluble transmembrane-only variant of MOR, namely wsMOR-G2, and the same variant but where the native MOR C-terminus is included, namely wsMOR-G2_Cter. In the latter case, no modifications to the wild-type sequence of the C-terminus were carried out. With these constructs, it is possible to demonstrate the specific role of the native receptor's C-terminus with the G-protein interaction with an electronic readout. The G-protein was expressed and purified as we reported previously [17].

B. Fabrication of GFET Arrays

Graphene was prepared on a piece of copper foil (99.8%, Alfa Aesar, Tewksbury, MA) by low-pressure chemical vapor deposition at 1020°C in a H_2(99.999% purity, 80 standard cubic centimeters per minute (sccm)) and CH_4(99.999% purity, 10 sccm) atmosphere. Graphene was then transferred via a bubbling transfer method [18] onto a 52-device electrode array, as shown in Fig 1a. The electrode arrays were pre-patterned on a Si wafer with 285 nm SiO_2. The SiO_2/Si wafer was spin-coated with PMGI (Kayaku Advanced Materials Inc., Westborough, MA) and S1813 photoresist (Kayaku Advanced Materials Inc.), followed by a standard photolithography process. The patterned wafer was developed by MF-319 developer (Kayaku Advanced Materials Inc.) for 45s. The contact metallization was 5nm Cr and 40 nm Au, deposited by thermal evaporation at a rate of 1 Å /s. Then the wafer was incubated in remover 1165 (Kayaku Advanced Materials Inc.) for liftoff. Another level of lithography process and O_2 plasma etching technique (0.8 Torr pressure; 60 W power; 30 s duration) were used to define a 100 μm$\times$10μm graphene channel. The remaining photoresist was stripped by remover 1165. Finally, the GFET arrays were annealed in an Ar/H_2 atmosphere (Ar flow rate 1000 sccm; H_2 flow rate 250 sccm) at 225 °C for 1 h to remove any residues from the photolithographic processing before use.

C. Functionalization of Graphene Surface With wsMOR

The immobilization of C-terminus-containing variant receptor, wsMOR-G2_Cter, on GFETs was done through the linker 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE, Sigma-Aldrich, St. Louis, MO) as indicated in Fig. 1b. GFET arrays were incubated in 1 mM PBASE in N, N-dimethylformamide (DMF, Fisher Scientific, Hampton, NH) for 20 h. A long incubation time is essential to achieve maximum coverage of PBASE on graphene. The aromatic pyrenyl group in PBASE binds to the graphene basal plane through $\pi - \pi$ stacking [19]–[21]. Next the GFETs were exposed to 2μg/ml of wsMOR-G2_Cter in 10 mM sodium phosphate (NaPi) buffer (pH=8.0, 0.01% SDS, sodium dodecyl sulfate) for 3 h. SDS is present in the buffer since it is needed during the protein purification process and it is not easily removed as we discussed previously [3]. The succinimide group in the PBASE molecule conjugates with an amine group in the protein to form a stable amide bond and immobilize the protein. A series of G-protein solutions with known concentrations in 10 mM NaPi buffer solution (pH=8.0, 0.01% SDS) were then added onto the GFET surfaces for 1h to allow for G-protein coupling. The arrays were completely rinsed by

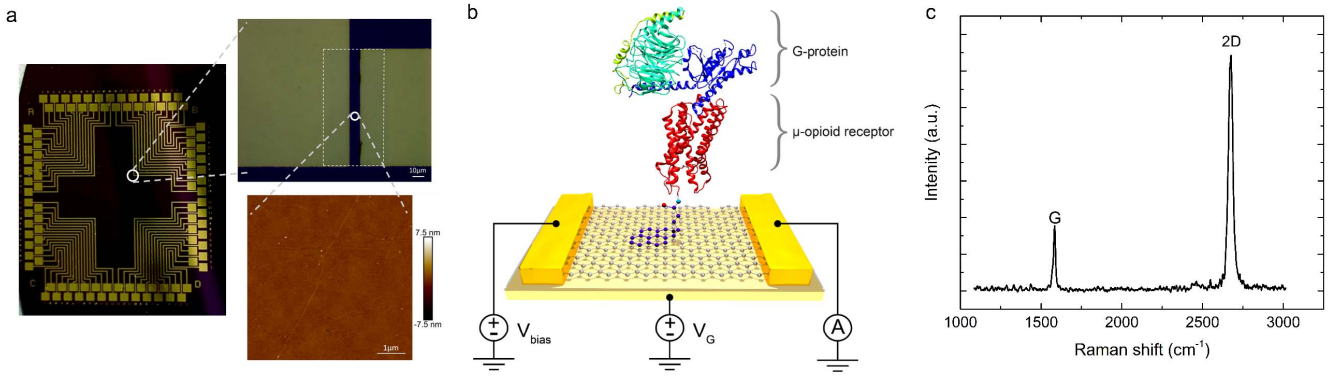

Fig. 1. (a) Left: A photo of one biosensor array. Right top: Optical microscope image of one GFET device. The white dot box indicates the region covered by graphene. Right bottom: AFM image of unmodified graphene in the channel region. (b) Schematic of GFET biosensor immobilized with wsMOR-G2 or wsMOR-G2_Cter through a 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE) linker. The wsMOR-G variants (red), either with or without the native C-terminus, were specifically linked with a G-protein, where the blue, turquoise and green regions indicate alpha (α), beta (β) and gamma (γ) subunits, respectively. (c) Raman spectrum of graphene monolayer.

deionized water to wash off any unbounded molecules and then N_2 blow-dried after each exposure to the solution.

D. Electrical Readout of the Biosensor and Data Fitting

The electrical readout of the sensor arrays was recorded after being exposed to wsMOR and G-protein, respectively. The current-gate voltage (I-V_g) characteristics of GFETs were measured in the dry state by using a probe station (MPS150, Cascade Microtech Inc., Beaverton, OR). The data was fitted by using non-linear least squares with the equation [10]:

$$I^{-1} = [e\alpha\mu(V_g - V_D)]^{-1} + I_s^{-1} \quad (1)$$

where I_S is the measured source-drain current; μ is the carrier mobility; V_g is the applied gate voltage; α is a constant relating gate voltage to carrier number density; V_D is the Dirac voltage and I_S is the saturation current due to short range scattering[18]. The unit of current I and I_s is ampere, A; unit of carrier mobility μ is $cm^2/(V{\cdot}s)$; unit of voltage V_g and V_D is volt, V; unit of constant α is $cm^{-2}V^{-2}$; unit of the elementary charge e is coulomb, C.

The Dirac voltage shift caused by G-protein coupling was taken as the sensor response, ΔV_D^{REL} and its relation with G-protein concentration was fitted by the Hill-Langmuir equation [23], [24]:

$$\Delta V_D^{REL} = A(c/K_A)^a/(1 + (c/K_A)^a) \quad (2)$$

where A is the magnitude of the saturated response; c is the concentration of G-protein; K_A is the G-protein concentration producing half occupation; and a is the Hill coefficient. The unit of the relative Dirac voltage shift ΔV_D^{REL} is V; unit of amplitude A is also V; unit of concentration c is mol/L or M; unit of K_A is also M. The Hill coefficient a has no unit.

III. Results

We used Raman spectroscopy to characterize the pristine graphene film. A large 2D/G peak ratio and no obvious D peak were observed, as shown in Fig. 1c, indicative of high-quality monolayer graphene [25]. Atomic force microscope (AFM) image of the channel area (Fig.1a) after the annealing process indicated a clean graphene surface with very little residue from previous processing steps. The immobilization of wsMOR-G2_Cter on graphene surface was examined by AFM and compared with wsMOR-G2, which only contains the transmembrane domain (*i.e.*, without the C-terminus), as shown in Fig. 2a-b. The white spots indicate the height and size of immobilized protein, with circled ones represent molecules in monomeric state. The height histogram of wsMOR macromolecules was obtained from the AFM data and well fitted by a Gaussian distribution. The mean height of wsMOR-G2_Cter is 4.57 ± 0.09 nm whereas that of the transmembrane only wsMOR-G2 is 4.21 ± 0.14 nm. Larger aggregations are also observed, but histogram calculations show that particles with a height larger than 10 nm only account for ~5% of the total number. These data indicate that both proteins have high purity as monomer (95%).

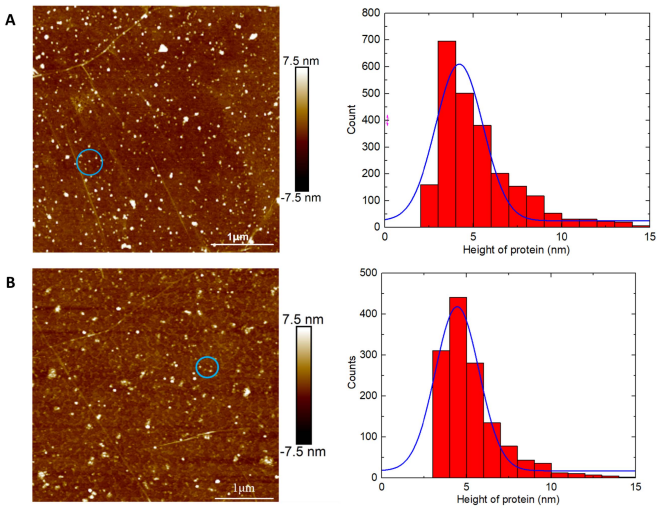

Fig. 2. AFM image and height histogram of (a) wsMOR-G2_Cter and (b) wsMOR-G2. The blue circle on AFM image indicates monomeric proteins. The height histogram is fitted by a Gaussian distribution (blue line).

To investigate the role of the C-terminus in G-protein association, we fabricated both wsMOR-G2 and wsMOR-G2_Cter

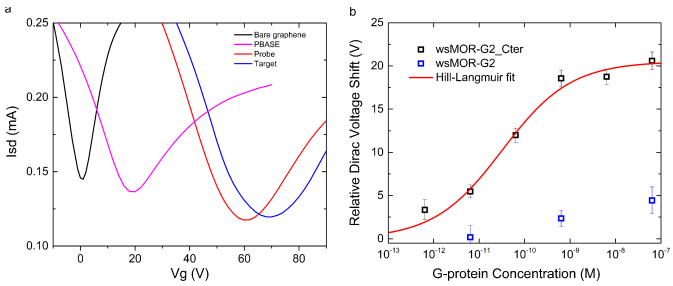

Fig. 3. (a) The I_{sd}-V_g characteristics for a graphene transistor which was annealed, functionalized with PBASE, immobilized with wsMOR-G2_Cter, and exposed to 6×10^{-8} M G-protein solution. (b) Relative Dirac voltage shift of a MOR-functionalized GFET sensor to solutions of varying concentrations of G-protein. The red curve is a fit based on the Hill-Langmuir equation. Blue data points are results of control experiments. The error bars represent the standard deviation of the mean.

functionalized GFETs and studied their binding performance against solutions of G-protein in 10 mM NaPi buffer solution (pH=8.0, 0.01% SDS). The size of GFET channel is 100 μm by 10 μm. There is no sample pool connected to the device. The target solution is directly pipetted onto the surface of the chip and covers the graphene channels. The source-drain current versus gate voltage (I_{sd}-V_g) characteristics of one typical GFET are demonstrated in Fig 3a. After each immobilization step, the sensor arrays showed a Dirac voltage shift, which is due to the chemical gating effect associated with the binding of target protein on the functionalized GFET surface [26]. The Dirac voltage shift, caused by G-protein binding, relative to that measured upon exposure to pure buffer solution was recorded as the sensor response, ΔV_D^{REL} and plotted in Fig. 3b. A high-quality fit was obtained, with the best fitting parameters $A = 20.5 \pm 1.0$ V, $a = 0.58 \pm 0.10$, and $K_A = 32.3 \pm 11.1$ pM. The responses of wsMOR-G2 functionalized GFET arrays against G-protein solution at three different concentrations (6 pM, 0.6 nM and 60 nM) were also measured, at a value of 0.17 V, 2.35 V and 4.43 V, respectively (Fig. 3b). In Fig. 3b, there are error bars for data points, which represent the standard deviation of the mean in each case. Each error bar was obtained across all the devices on one chip. The small standard deviation in pM range indicates a high reproducibility across 50 devices.

IV. Discussion

The development of novel opioid pain medications devoid of major side effects has been slow due to a lack of understanding of the molecular pharmacology of opioid receptor interactions with G-protein and its related signaling pathway. The lipid bilayer has been a huge hurdle in advancing understanding of the molecular pharmacology of the G-protein interaction with GPCR. Our group has been developing an approach using wsMOR, to make a relatively simple system to investigate the pharmacology of MOR, which is the key receptor related to the pain signaling pathway. We have successfully demonstrated that opioid recognition and binding are well preserved in variants of wsMOR in the absence of a lipid bilayer. In this study we demonstrated that the lipid bilayer is not essential to the G-protein recognition and binding. Thus, we open a new door towards investigating and understanding the molecular signaling pathway for MOR, and potentially for other receptors in the GPCR family.

The AFM data reveals that both variants of wsMOR have a high density of $\sim$100 μm^{-2}, in accordance with our previous report [27], indicating the successful immobilization on a graphene surface. The mean height of both proteins obtained by Gaussian analysis is in good agreement with the molecular dimensions of the monomeric protein. These findings make it clear that both proteins are purified successfully in a monomeric state. It was also found that the mean height of wsMOR-G2_Cter is slightly larger than that of wsMOR-G2, which can be ascribed to the presence of the native MOR C-terminus. The successful purification of wsMOR-G2_Cter in buffer indicates that the native C terminus did not change the solubility of the receptor. wsMOR-G2_Cter provides a novel tool to investigate the role of C terminus in the receptor function as compare to the full-length variants and the transmembrane domain versions as we have presented in the past [2], [3].

The electrical response of the wsMOR-functionalized GFETs against G-protein is demonstrated in Fig. 3. Each data point was obtained from a single 52-device chip. The short error bars indicate little variance across the devices in one biosensor array. The relation between sensor response and G-protein concentration was well fitted with the Hill-Langmuir equation (Fig. 3), which directly shows the coupling of the G-protein with the wsMOR-G2_Cter variant. The small value of K_A indicates the high affinity of the G-protein for wsMOR-G2_Cter on a label-free graphene platform. To further confirm the role of the C-terminus in G-protein coupling, we measured the responses of wsMOR-G2 functionalized sensor against G-protein solution at three different concentrations. The sensor responses ΔV_D^{REL} were several times smaller than those of the wsMOR-G2_Cter functionalized sensors at the same concentrations. This smaller shift is essentially observed in the absence of G-protein, indicating little or no binding of G-protein to the receptor variant on the graphene surface at this G-protein concentration. This sensing result proves that the C-terminus is a critical structural element for mediating the coupling of the wsMOR-G2_Cter variant with the respective G-protein. Without the C-terminus, the G-protein will be unable or difficult to couple with MOR.

From our results, we propose that the GFET biosensing method is a promising detection tool for investigating the binding properties of wsMOR-G2_Cter. Particularly, we envision the ability to dissect the relationship between the ligand binding and the G-protein binding, which will help to understand the molecular mechanism of G-protein-coupled receptors (GPCR).

The major limitation of this study is that we did not introduce any opioid ligand into the system to investigate the dynamics of G-protein interactions. We are planning to do this in the next stage of development of this novel technology. Mutagenesis can also be performed to investigate the key residues in the binding pocket that control the signaling pathway for the G-protein interaction. This will be the basis of additional future studies. The sensors are quite uniform

and reproducible from device to device, but we have not conducted experiments to quantify their stability over time. Since they contain a protein functionalization layer, at the least they would require storage conditions that would protect the biomolecule.

V. Conclusion

In conclusion, the lipid environment is not essential for the G-protein interaction with MOR, however, the C terminus of MOR is essential for G-protein recognition, high affinity binding, and subsequent pharmacological signaling. A novel system to detect MOR-G protein interaction in pM sensitivity is developed using wsMOR variants. The novel technologies developed in this study can be used to further reveal the molecular pharmacology and signaling pathway of the G-protein interactions with MOR. The newly designed wsMOR-G2_Cter provides a novel tool to investigate the role of C terminus in the signaling pathway.

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