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All-Electronic Quantification of Neuropeptide—Receptor Interaction Using a Bias-Free Functionalized Graphene Microelectrode

Jinglei Ping[†], Ramya Vishnubhotla[†], Jin Xi[‡], Pedro Ducos[†], Jeffery G. Saven[§], Renyu Liu^{*,‡}, and Alan T. Charlie Johnson^{*,†}

[†]Department of Physics and Astronomy, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

[‡]Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

[§]Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

Abstract

Opioid neuropeptides play a significant role in pain perception, appetite regulation, sleep, memory, and learning. Advances in understanding of opioid peptide physiology are held back by the lack of methodologies for real-time quantification of affinities and kinetics of the opioid neuropeptide–receptor interaction at levels typical of endogenous secretion (<50 pM) in biosolutions with physiological ionic strength. To address this challenge, we developed all-electronic opioid–neuropeptide biosensors based on graphene microelectrodes functionalized with a computationally redesigned water-soluble μ -opioid receptor. We used the functionalized microelectrode in a bias-free charge measurement configuration to measure the binding kinetics and equilibrium binding properties of the engineered receptor with [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin and β -endorphin at picomolar levels in real time.

Abstract

*Corresponding Authors renyu.liu@uphs.upenn.edu., cjohnson@physics.upenn.edu.

ASSOCIATED CONTENT

Supporting Information

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Functionalization density as a function of incubation time; estimate of the density of immobilized wsMOR; detailed methods for transistor-based sensor measurements (PDF)

The authors declare no competing financial interest.

Keywords

neuropeptides; graphene; microelectrode; bias-free; biosensors; affinity; kinetics

Endogenous opioid neuropeptides are an important class of neurotransmitters that play a critical role in stress response, analgesia, addiction, pain management, and cardiovascular control.¹ The dysregulation of endogenous opioid neuropeptides may result in neurologic and psychiatric disorders such as depression, borderline personality disorder, as well as Alzheimer's, Huntington's, and Parkinson's.^{2–4} Thus, understanding opioid–neuropeptide physiology and the downstream applications in pharmacology,⁵ anesthesia and surgery,⁶ and therapeutics⁷ is highly significant. Conventional voltammetry methods can be used to real-time detect neuropeptides, with relatively low sensitivity, >100 nM.⁸ Quantification of opioid neuropeptides at low levels typical in human plasma (<50 pM),^{9, 10} a crucial enabling step in neuropeptide investigation, currently relies on liquid chromatography and n-rounds mass spectroscopy (LC-MSⁿ), which is incompatible with real-time analysis. This method is highly invasive as the biofluid must be sampled for analysis, and it suffers from low spatial resolution, low throughput, and high cost.¹¹ Another emerging technique for neuropeptide quantification, biotransistors based on low-dimensional nanomaterials such as silicon nanowires¹² and graphene,¹³ is not suitable for use *in vivo* or in ionic complex biofluids due to limitations on sensitivity caused by the Debye screening effect.¹⁴

Here, we demonstrate all-electronic real-time recording of neuropeptide–receptor interactions in solution with physiological ionic strength (~ 150 mM) using graphene micro-electrodes^{15–17} functionalized with an engineered water-soluble μ -opioid receptor (wsMOR).^{13,18,19} The wsMOR is a computationally redesigned variant of the membrane protein that, unlike the native MOR, is stable in aqueous solution. The target molecules for our experiments were [D-Ala², N-MePhe⁴, Glyol]-enkephalin and β -endorphin, both opioid peptides with high specificity for the native MOR. Conventional electro-chemical methods, which include one or more additional electrodes to apply a voltage bias and/or to measure

the potential of the solution, may lose sensitivity at low target concentration due to nonspecific adsorption on the additional electrodes.²⁰ To overcome this limitation, our approach is to monitor the spontaneous, zero-bias Faradaic charge transfer from the solution into the microelectrode ($\sim$ pC/s).^{15,16} We show that this current varies systematically with the analyte concentration, providing picomolar sensitivity in a solution with physiological salt content. We used this approach to quantify the association rate constant, $2.8 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, and the dissociation rate constant, $0.089 \pm 0.001 \text{ min}^{-1}$, for binding between the engineered wsMOR and enkephalin. The Faradaic current measured after the electrode equilibrated with the solution varied systematically with the concentration of the target analytes, enkephalin and β -endorphin, with a limit of detection at the picomolar level. This methodology offers a pathway toward *in vivo* quantification of neuropeptide secretion with high sensitivity and spatiotemporal resolution for interrogating and understanding neurophysiological and biopsychological effects of neuropeptides.

RESULTS AND DISCUSSION

The experimental setup for Faradaic charge measurement¹⁵ is shown in Figure 1a (see Methods for more information regarding the measurement). All measurements were conducted in full-strength phosphate buffer solution ($1\times$ PBS). Faradaic charge transfer occurred through the series combination of the solution diffuse layer resistance ($\sim 10 \text{ k}\Omega$),²¹ the graphene–solution interface charge transfer resistance ($R_{\text{ct}} \sim 100 \text{ G}\Omega$),¹⁵ the sheet resistance of graphene ($\sim 10^2\text{--}10^3 \text{ }\Omega/\square$), and the graphene–gold contact resistance ($\sim 10 \text{ }\Omega$).²² The charge transfer rate, $\sim$ pC/s, was thus primarily determined by the charge transfer resistance R_{ct} , which is independent of ionic strength.¹⁶ Charge transferred from the solution to graphene accumulated on the feedback capacitor, C_f , of the electrometer to produce the readout voltage. For graphene microelectrodes, the effective potential that drives the spontaneous Faradaic current decays *logarithmically* with increasing ionic strength,¹⁵ instead of the much faster *exponential* decrease that is characteristic of transistor-based devices. The charge transfer signal was, for this reason, readily detected at physiological ionic strength. As discussed below, our observations are consistent with the view that target binding modulates charge transfer through physical blocking of charge transfer sites.

For fabrication of graphene-based microelectrode arrays, large-area graphene sheets were prepared by chemical vapor deposition and transferred using a low-contamination bubbling method²³ onto a Si/SiO₂ substrate with prefabricated 45 nm thick Cr/Au electrodes. Graphene microelectrodes ($50 \text{ }\mu\text{m} \times 50 \text{ }\mu\text{m}$) were then defined with photolithography and plasma etching, and the metal contacts were passivated by a hard-baked, $\sim 20 \text{ }\mu\text{m}$ thick SU-8 (MicroChem) layer so that charge transfer could occur only at the graphene–solution interface (see Methods for further details of the fabrication process).

The graphene microelectrodes were functionalized with the computationally redesigned wsMOR. The functionalization process parameters (i.e., solution concentrations and incubation times) were optimized to maximize the density of immobilized wsMOR. One key factor was a relatively long incubation time ($>20 \text{ h}$) in a 1 mM solution of the linker molecule 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBASE, Sigma-Aldrich) in methanol, a crucial enabling step for high-quality functionalization (see Supporting

Information section 1 for details). PBASE is a bifunctional linker with an aromatic pyrenyl group that irreversibly adsorbs onto the basal plane of graphene through a noncovalent π - π interaction.^{24,25} The PBASE-covered microelectrodes were next exposed to wsMOR (3 $\mu\text{g/mL}$) in $1\times$ PBS. The succinimide groups in the PBASE layer conjugated with primary amine groups on the surface of wsMOR molecules to form stable amide bonds that immobilized wsMOR. The process led to a high density of PBASE and wsMOR on the graphene surface,^{15,26} which appeared as a uniform brush-like layer when imaged by atomic force microscopy (AFM), as shown in Figure 1c. The apparent height of wsMOR as determined from AFM line scans (Figure 1d) was ~ 4.3 nm, in good agreement with expectations given the wsMOR mass of 46 kDa.¹⁴ We used the measured Dirac voltage shift induced by wsMOR binding and the expected charge state of the wsMOR to estimate the density of the wsMOR functionalization at $550 \mu\text{m}^{-2}$ (details are provided in the Supporting Information, section 2). This value is smaller than that in our earlier report²⁷ of $1300 \mu\text{m}^{-2}$ for single-stranded DNA 22-mers immobilized on graphene using a very similar procedure, which is attributed to the effect of the larger size of the wsMOR on the surface packing efficiency.

Before performing graphene microelectrode measurements of the real-time binding properties of wsMOR in ionic solution, we characterized the equilibrium dissociation constant and verified specific binding between wsMOR and enkephalin by using biosensors based on wsMOR-functionalized graphene field-effect transistors (GFETs).¹³ The experimental details are provided in the Supporting Information, section 3. Typically, a biosensor response R against the target at concentration c is described by the Hill–Langmuir equation:²⁸

$$R = A \frac{(c/K_A)^n}{1 + (c/K_A)^n} \quad (1)$$

where A (1) where A is the magnitude of the sensor response, K_A is the concentration producing half occupation, and n is the Hill coefficient. We measured the current–gate voltage (I – V_G) characteristics of the wsMOR/GFETs in the dry state and quantified the sensor response R as the shift of the Dirac voltage, V_D , which varied systematically with the concentration of enkephalin in a low-salt buffer ($0.002\times$ PBS). As shown in Figure 1e,

V_D was well fit by eq 1, with the fit parameters $A = 4.4 \pm 0.1$ V, $K_A = 0.79 \pm 0.10$ nM, and $n = 0.6 \pm 0.1$. The fit value for the value of K_A was in very good agreement with the value of 1.2–3.1 nM obtained by optical assays for the binding between native MOR and enkephalin.^{29,30} The best fit value for the Hill coefficient n was smaller than the value obtained from radioligand binding assays, ~ 1.0 ,³¹ but consistent that in our earlier report for DNA hybridization on graphene, 0.56 ± 0.07 ,²⁷ which is ascribed to the interaction between graphene, the receptor, and the analyte (wsMOR and enkephalin in this case). We also tested the GFET biosensor response against oxytocin, an endogenous neuropeptide that is known not to bind specifically to native MOR.³² The response of the biotransistors to 1 nM oxytocin solution was very small, 0.17 V (Figure 1e), indicating a low level of nonspecific binding of oxytocin to the wsMOR. We note that the transistor-based biosensor, although

suitable for quantifying the enkephalin–receptor dissociation constant, suffers from the Debye screening effect,¹⁴ which required that the target be presented in a low-salt buffer solution for real-time testing.

Now we discuss real-time measurements of the neuro-peptide–wsMOR interaction using the graphene microelectrodes. The graphene microelectrode was exposed to 1× PBS at full ionic strength (~150 mM). To determine the sensor response to enkephalin at different concentrations, drops of enkephalin solution at successively larger concentrations were placed onto the device; after each drop was applied, the feedback capacitor of the electrometer was discharged, and then a charge transfer time trace was acquired. Time traces of the Faradaic charge transfer, $Q(t)$, for three different concentrations are shown in Figure 2a, with the corresponding Faradaic currents, $i(t) = dQ/dt$, shown in Figure 2b. From Figure 2b, we see that the Faradaic current for 0.2 pM enkephalin decreased gradually over tens of minutes and saturated after more than 30 min, which is ascribed to an increase in R_{ct} caused by enkephalin binding to wsMOR on the graphene microelectrode so that charge transfer sites were physically blocked.¹⁶ In contrast, for a target concentration of 22 nM, the current decreased much more quickly and saturated after about 6 min. To quantify the binding kinetics, we fit the current $i(t)$ with a single-time relaxation model³³ describing ligand–receptor binding:

$$i(t) = i_A(c) - i_B(c)(1 - e^{-t/\tau}) \quad (2)$$

where $i_A(c)$ is an offset current at $t = 0$, τ is the saturation time constant, and $i_A(c) - i_B(c)$ is the saturation current for long times as the system reaches equilibrium (for practical purposes, this requires $t \gg 3\tau$). For 0.2 pM enkephalin, the best fit value is $\tau = 11.2 \pm 0.7$ min, whereas for 22 nM, $\tau = 1.40 \pm 0.02$ min, a factor of about 8.0 times smaller.

For ligand–receptor binding, the observed saturation rate $k_{ob} = 1/\tau$ depends on the target concentration c through the relation $k_{on} = (k_{ob} - k_{off})/c$, where k_{on} and k_{off} are the association rate constant and the dissociation rate constant, respectively.³³ The measurement presented in Figure 2c, is well described by this relationship, and the best fit values for k_{on} and k_{off} are $2.8 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ and $0.089 \pm 0.001 \text{ min}^{-1}$, respectively. These values are in good agreement with those determined using radioligand binding assays: $k_{on} = 8.5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$,³⁴ and $k_{off} = 0.155 \pm 0.001 \text{ min}^{-1}$.³⁵ The corresponding dissociation constant $K_A = k_{off}/k_{on}$, is $3.1 \pm 0.2 \text{ nM}$, slightly higher than the value derived from the FET sensor measurement, $0.79 \pm 0.10 \text{ nM}$, but in good agreement with that found using optical assays, 1.2–3.1 nM,^{29,30} for native MOR and enkephalin binding.

To investigate the use of the functionalized graphene microelectrode as an enkephalin biosensor, the limiting value of Faradaic current as $t \rightarrow \infty$, $i_A(c) - i_B(c)$, was obtained by fitting the measured charge transfer to eq 2, and the sensor response was taken to be a relative Faradaic current compared to that measured when the microelectrode was exposed to pure PBS. As shown in Figure 3, the variation of the relative Faradaic current with enkephalin concentration was well fit by the Hill–Langmuir formula eq 1, with high signal-to-noise ratio. The best fit values of the parameters are the amplitude $A = -0.56 \pm 0.01 \text{ pA}$,

the concentration producing half occupation, $K_A = 3.6 \pm 0.4$ nM (in excellent agreement with the value obtained by kinetic measurements discussed above), and the Hill coefficient, $n = 0.5 \pm 0.1$, again reduced below 1.0 by interactions between graphene, the receptor, and the enkephalin target.

We applied the same methodology to β -endorphin, a second neuropeptide known to bind to native MOR. As demonstrated in Figure 3, the neuropeptide biosensor response varied systematically with target concentration with high signal-to-noise ratio and again could be well fit by eq 1. The best fit value for the dissociation constant was $K_A = 1.5 \pm 0.2$ nM, in good agreement with the value obtained via radioligand arrays, 2–6 nM;³⁶ the value for n was 0.6 ± 0.1 , in the range typical of functionalized graphene sensors. We found that the best fit value for the amplitude $A = -0.89 \pm 0.03$ pA for β -endorphin is ~ 1.6 times larger than the amplitude found for enkephalin. This is ascribed to greater inhibition of charge diffusion^{37,38} by β -endorphin compared to that of enkephalin due to the former's greater molecular weight and thus size. It is possible that the signal strength for the microelectrode sensor depends on multiple attributes of the target, such as size, chirality, and charge, making this an interesting topic for future exploration. Remarkably, for both enkephalin and β -endorphin, the excellent signal-to-noise performance leads to a detection limit in the picomolar range, suggesting this sensor system is capable of resolving the mean secretion level of enkephalin (~ 15 pM)⁹ or β -endorphin (~ 40 pM)¹⁰ characteristic of human plasma.

To evaluate the reproducibility of the opioid–neuropeptide biosensors, we tested six different devices for enkephalin and two devices for β -endorphin. The device-to-device variation in the response amplitude was $\pm 15\%$, whereas the dissociation constants for both targets were consistent at $\pm 10\%$. We also investigated the selectivity of the wsMOR-functionalized neuropeptide biosensors by testing against oxytocin as a negative control (Figure 3). The response of the biosensor to oxytocin was essentially negligible at concentrations below 1 μ M, providing strong evidence that the sensor response reflects specific binding of the target and wsMOR.

CONCLUSIONS

We demonstrated the use of graphene microelectrodes functionalized with a soluble variant of the human MOR for neuropeptide detection with high sensitivity (picomolar level) and specificity in biofluids with physiological ionic strength. The kinetics and equilibrium binding properties of two neuropeptides, [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin and β -endorphin, were investigated and quantified. The results were in excellent agreement with benchmarks set by conventional radioligand and optical assays, as well as neuropeptide biosensors based on field-effect transistors that were measured in the dry state. The size of the active sensor region for this work was $50 \mu\text{m} \times 50 \mu\text{m}$, on the scale of larger neurons, but this could be reduced to the scale of the smallest neurons ($\sim 4 \mu\text{m}$) using optical lithography. The measurement time was ~ 30 min, which enabled determination of the relevant fitting parameters with high precision. If a microelectrode sensor was precalibrated so the time constant for a given concentration was known, then it might be possible to determine the concentration more rapidly by simply fitting the first few minutes of the time trace (Figure 2). These all-electronic biosensors are therefore potentially suitable for development for use

in *in vitro* clinical testing or implantable systems for interrogating neuropeptide secretion with high spatial and time resolution.

METHODS

Graphene Growth.

Copper foil (99.8% purity) was loaded into a 4 in. quartz tube furnace and annealed for 30 min at 1050 °C in an ultrahigh purity (99.999%) hydrogen atmosphere (flow rate = 80 sccm; pressure of 850 mT at the tube outlet) for removal of oxide residues. Graphene was then deposited by low-pressure chemical vapor deposition using methane as a precursor (flow rate = 45 sccm, growth time of 60 min).

Graphene Device Fabrication.

The graphene–copper growth substrate was coated with a 500 nm layer of poly(methyl methacrylate) (PMMA, MICROCHEM), and the PMMA–graphene film was floated off the surface by immersion in a 0.1 M NaOH solution with the graphene–copper growth substrate connected to the cathode of a power supply. The PMMA–graphene film was transferred onto a silicon wafer with an array of 5 nm/40 nm Cr/Au contact electrodes that was previously fabricated using photolithography. After removal of PMMA with acetone, the graphene film was cleaned by annealing at 250 °C in 1000 sccm argon and 400 sccm hydrogen for 1 h.

For the FET-based devices, $2\ \mu\text{m} \times 10\ \mu\text{m}$ graphene channels were defined by photolithography (photoresist S1813, MICROCHEM) and oxygen plasma etching. For the graphene microelectrodes, $50\ \mu\text{m} \times 50\ \mu\text{m}$ graphene electrodes were defined by photolithography (photo-resist AZ 5214 E, MICROCHEM) followed by oxygen plasma etching. A layer of photoresist SU-8 (MICROCHEM) was then applied to the device, and the passivation layer covering the electrodes was defined by photolithography.

Graphene Functionalization.

Graphene electrodes were incubated in 1 mM 1-pyrenebutyric acid N-hydroxysuccinimide ester solution in ethanol for 20 h and then washed thoroughly with methanol and deionized water to fully remove residual solute and solvent. Next, the devices were incubated in 3 $\mu\text{g}/\text{mL}$ wsMOR solution in PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate, pH 7.4) for 1 h for protein immobilization. After wsMOR functionalization, the PBASE that had not bound to wsMOR was passivated by exposing the chips to PBS with pH 8.6 for 1 h, much longer than the half-life of PBASE at this pH (~10 min).³⁹

Charge Transfer Measurement Using Graphene Micro-electrodes.

The noninverting input of the operational amplifier in an electrometer (Keithley 6517a) was grounded. The inverting input was connected to the graphene microelectrode, which connected the microelectrode to a virtual ground, so all charge transferred into the microelectrode was delivered to the feedback capacitor C_f in Figure 1a to produce the measured voltage readout. To start the measurement, the microelectrode was exposed to a solution of wsMOR at known concentration in $1\times$ PBS (137 mM sodium chloride, 2.7 mM

potassium chloride, 10 mM phosphate, pH 7.4), and a time trace of the sensor response was taken immediately.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Schematic of the electronic setup for measurement of charge transfer from a biosolution to a graphene microelectrode functionalized with water-soluble μ -opioid receptor (wsMOR). (b) AFM image of monolayer graphene on SiO_2/Si . (c) AFM image of wsMOR-functionalized graphene on SiO_2/Si . (d) Line scans indicated in panels b and c, showing the change in apparent height due to functionalization with wsMOR. (e) Response of a graphene transistor-based neuropeptide biosensor to solutions of varying concentration of [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin in low-salt buffer (0.002× PBS). Red curve is a fit to a model based on the Hill–Langmuir equation. The green data point is the result of a negative control experiment against oxytocin.

Figure 2.

(a) Real-time Faradaic charge transfer into graphene for 0.2 pM (blue), 2.2 nM (green), and 22 nM (yellow) [D-Ala², NMePhe⁴, Gly-ol]-enkephalin in phosphate buffer solution. (b) Extracted Faradaic current as a function of time. The red curves in both panels are exponential fits characterized by a single time constant τ , as discussed in the main text. (c) Plot of the inverse of the time constant ($k_{ob} = 1/\tau$) as a function of concentration. The red line is a linear fit to the data.

Figure 3. Relative Faradaic current for the [D-Ala², N-MePhe⁴, Glyol]-enkephalin, β -endorphin, and oxytocin as a function of concentration. The error bars (<femtoamp level) are smaller than the data points.