Electronic Supplementary Information

of

Functionalized Two-Dimensional Nanochannel Membranes to Distinguish Methylated/Unmethylated Peptides for Sensing Cellular G9a Protein

Jing-Jing Hu[1‡], Niya Lin[1‡], Wenlian Jiang[1], Rui Liu[1], Xiaoding Lou[1,2*], Fan Xia[1,2*]

[1] State Key Laboratory of Geomicrobiology and Environmental Changes, Engineering Research Center of Nano-Geomaterials of Ministry of Education, Faculty of Materials Science and Chemistry, China University of Geosciences, Wuhan 430074, China

[2] Shenzhen Research Institute of China University of Geosciences, Shenzhen 518057, China

‡ These authors contributed equally to this work.

*Corresponding Author(s): Xiaoding Lou: louxiaoding@cug.edu.cn; Fan Xia: xiafan@cug.edu.cn

Table of Contents

Materials and measurements

Single-layer graphene oxide (GO) powder membrane was obtained from Jiangsu XFNANO Materials Tech Co., Ltd. The peptides, MARTKQTA(named as MA8), MARTK(me)QTA (named as MA8me1), MARTK$(me)_2$QTA (named as MA8me2), MARTK$(me)_3$QTA (named as MA8me3), TARKSTG (named as TG7), and TARK$(me)_2$STG (named as TG7me2) were customized from GL Biochem Ltd. (Shanghai, China). 4'-aminobenzo-18-crown-6 was obtained from Sigma-Aldrich (Shanghai) Trading Co., Ltd. 0.22 μm microfiltration membrane was obtained from Shanghai Xingya Purification Materials Factory. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Beijing Labgic Technology Co., Ltd. Phenylmethanesulfonyl fluoride (PMSF) was purchased from Beyotime Biotechnology. Sodium chloride and magnesium chloride were purchased form Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Recombinant human histone-lysine N-methyltransferase protein (G9a) was bought from AntibodySystem SAS. Human G9a enzyme-linked immunosorbent assay (ELISA) kit was purchased from Wuhan Fine Biotech Co., Ltd. Bovine serum albumin (BSA) and S-adenosylmethionine (SAM) were bought from Shanghai Aladdin Biochemical Technology Co., Ltd. α-Chymotrypsin, phosphatase and UNC0638 inhibitor were obtained from MedChemExpress. Human normal mammary epithelial cells (MCF-10A), human breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-468), and MCF-10A cell-specific culture medium was purchased from Wuhan Pricella Biotechnology Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate buffer saline (PBS, pH 7.4) were all obtained from HyClone Thermofisher (Beijing, China). All other reagents were obtained from commercial sources and used without further purification. Deionized water (18.2 MΩ·cm) used in all experiments was purified with a Heal Force water purification system (Shanghai, China).

Atomic force microscope (AFM, Multimode 8) and scanning electron microscopy (SEM, Hitachi, SU8010) were used to observe the morphology and structure of nanochannels. Electrospray ionization mass spectrometry (ESI-MS) was recorded on a Thermo Scientific Q Exactive mass spectrometer system. The Zeta potential of the peptides and dispersion liquids were measured on a Zeta potentiometer (Malvern Instruments, Nano-ZS 90). The I-V curve of the nanochannels was recorded by an electrochemical workstation (Shanghai Chenhua, CHI660E). The UV-Vis absorption spectrum was acquired on the UV/VIS spectrometer (Shimadzu Corporation, UV-2600). The absorption values of the ELISA test results were detected by a microplate reader (Tecan Trading AG, Switzerland). The culture environments with different oxygen concentrations were provided by hypoxic chambers (Precision BioMedicals Co., Ltd., 504001).

Construction of different membranes

2.5 mg/mL single-layer GO powder was dispersed in ultra-pure water. After magnetic stirring for 24 h, the solution was added with 50 mg/mL 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 30 mg/mL N-hydroxysuccinimide (NHS). After reaction for 4 h, 10 mg/mL 4'-aminobenzo-18-crown-6 (abbreviated to Crown) was added to the system and then react for 24 h to obtain the GO-Crown. 1 mL GO-Crown dispersion was taken out and assembled into a GO-Crown membrane by vacuum filtration. The filter membrane with a pore size of 0.45 μm and a diameter of 25 mm was used as the substrate. In order to obtain a stable membrane, the GO-Crown membrane was dried in a drying oven at 60 ^{o}C for 2 h after the filtration was completed, and the prepared GO-Crown membrane was peeled off from the substrate. GO membrane was prepared using the similar procedures.

The composite membrane was constructed by following procedures. 0.5 mL 2.5 mg/mL GO dispersion was taken out and assembled into a GO membrane by vacuum filtration. When the GO membrane was drawn into shape, 0.5 mL 2.5 mg/mL GO-Crown dispersion was taken out and assembled into a GO-Crown membrane above the GO membrane. The GO/GO-Crown composite film was obtained after the drying and peeling process.

The isothermal titration calorimetry (ITC) experiment

ITC experiment was used to evaluate the interaction between crown probe and peptides, including MA8, MA8me1, MA8me2 and MA8me3, using a VP-ITC microcalorimeter (MicroCal Inc.) at 25 ^{o}C. The crown probe (0.5 mM) was used as titration solution and the peptides (20 μM) were used as work solution. The peptide was loaded into the syringe, and titrated into the calorimetric cell containing the crown probe solution. The reference cell was filled with ultrapure water. The titration sequence consisted of a single 3 μL injection followed by a series of 10 μL solution, with a time interval of 100 s between injections to ensure that the thermal power returns to the baseline before the next injection. The stirring speed was 500 rpm. Titration experiments of methylated and unmethylated peptides were carried out under the same experimental conditions.

Electrically driven methylated or unmethylated peptides and measurements of the I-V curve

The functionalized GO-Crown membrane was interposed between the electrolytic cell, and the effective penetration area of the membrane was 0.2 mm. 2 mL NaCl electrolyte (1 mM) was added to one side of the cell (named as receiving

chamber), and 1.9 mL NaCl electrolyte (1 mM) was added to the other side (named as driving chamber). A pair of Ag/AgCl electrodes were respectively inserted into the two cells. Then the methylated or unmethylated peptides dissolved in 1 mM NaCl was added to the driving chamber. The current-time (I-T) mode of electrochemical workstation was chosed, -1 V voltage was applied between the two cells. After the electrical driving, the Linear Sweep Voltammetry (LSV) mode of electrochemical workstation was selected. The I-V test (voltage -0.2 V~ +0.2 V) was carried out immediately. Besides, all the tests were conducted at room temperature and at least three nanochannels of each sample were recorded to obtain the average value of I-V curve.

Evaluating the peptide responsiveness of 2D nanochannel membrane

To discuss the sensing property of the 2D nanochannel, a series of MA8 peptide with different concentrations (10 fM, 1 pM, 100 pM, 10 nM, 1 μM) dissolved in 1 mM NaCl were added to the driving chamber. The I-T mode of electrochemical workstation was chosed, -1 V voltage was applied between the two cells. After the electrical driving, the LSV mode of electrochemical workstation was selected. The current signals were recorded immediately.

To determine the best electrical driving time, the I-V test (voltage -0.2 V~ +0.2 V) was carried out immediately after a certain period of driving time (15 min-10 h) at -1 V voltage. The current signals were recorded immediately.

Drop coating MA8 peptide

2 μL of MA8 unmethylated peptide solution or methylated peptide solution with a certain concentration were absorbed by pipette, and then dripping onto GO-Crown or GO membrane. Ensuring the membrane was fully coated with peptide solution and incubated at room temperature for 12 h for the subsequent ion current testing.

G9a enzymatic reaction

50 μM SAM, 10 mM Tris-HCl buffer (pH 8.6) containing 0.1 mM $MgCl_2$, 10 μM TG7 and 20 nM G9a were incubated at room temperature for 6 h. After the reaction was completed, 100 μL of reaction liquid was added to the driving chamber that containing 1.9 mL 1 mM NaCl electrolyte, and 2 mL NaCl electrolyte (1 mM) was added to one side of the cell. The I-T mode of electrochemical workstation was chosed, -1 V voltage was applied between the two cells. After the electrical driving, the LSV mode of electrochemical workstation was selected and the I-V test (voltage -0.2 V~ +0.2 V) was carried out immediately.

Determination of the best enzymatic reaction time

To confirm the best enzymatic reaction time, the reaction liquid after certain period of enzymatic reaction time (0 h-7 h) was added to the cell, the I-V test was carried out immediately after being driven at a voltage of +1 V for 1 h.The current signals were recorded immediately.

Specificity of nanochannel for G9a

A series of proteinases including G9a, α-chymotrypsin, phosphatase, bovine serum albumin, and G9a inactivation were selected to verify the selectivity of GO-crown nanochannel. The concentration of each proteinase was 20 nM. The solution containing G9a was heated at 95 °C for 2 h to prepare the G9a inactivation group. The other reaction conditions and test conditions of other proteases were consistent with that of G9a.

Cell culture

MCF-7, MDA-MB-231, MDA-MB-468 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin streptomycin. MCF-10A cells were cultured in MCF-10A cell-specific culture medium. Both MCF-7, MDA-MB-231, MDA-MB-468 cells and MCF-10A cells were incubated in Petri dishes at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell lysis procedure

The culture dish with a growth cell density of about 90% was taken out from the incubator, the medium was sucked away, and the cells were washed with PBS for 3 times. Then the cells were placed on ice and scraped off with a cell scraper. Then the cell suspension was mixed well with a pipette, 10 μL cell suspension was taken, and the number of cells cultured in the petri dish was counted with a hemocytometer. Then the cell suspension was centrifuged at 1000 rpm for 4 min, the supernatant was discarded, the cell lysate was added and gently sucked several times to completely lysate the cell and get the protein. An ultrasonic cell crusher was then used to further crack the DNA goo in the cells.

Dectetion of the G9a from cell lysate

50 μM SAM, 10 mM Tris-HCl buffer (pH 8.6) containing 0.1 mM $MgCl_2$, 10 μM TG7 and cell lysate were incubated at room temperature for 6 h. After the reaction was completed, 100 μL of reaction liquid was added to the driving chamber

that containing 1.9 mL 1 mM NaCl electrolyte, and 2 mL NaCl electrolyte (1 mM) was added to one side of the cell. The I-V test was carried out immediately after being driven at a voltage of +1 V for 1 h. Besides, the cellular G9a expression was also mearsured by Human G9a ELISA kit. The operationswere carried out according to the instruction manual.

Inhibitor treatment

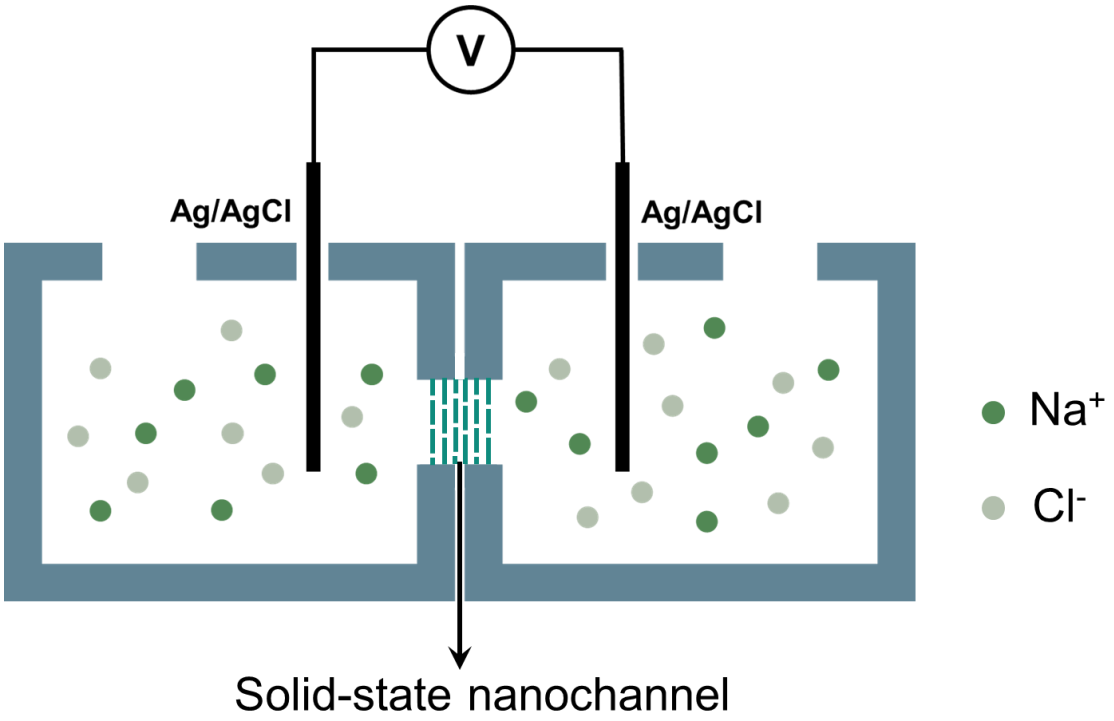
Different concentrations of UNC0638 inhibitors (15 nM, 150 nM, 1.5 μM) dissolved in DMSO were added to the normal growth petri dishes with cell density of about 70-80%, and the cells were lysed after co-culture for 72 h.

Hypoxia culture

According to the instructions for the hypoxia chamber, the petri dish with a density of about 70-80% of the growth cells was taken out from the incubator and placed into a hypoxia chamber containing a petri dish (without a lid) with 10-20 mL of sterile water. Special hypoxic gas (1% oxygen, 5% carbon dioxide, 94% nitrogen) was injected into the chamber at the rate of 20 liters of gas per minute. After 4 min, the ventilation valve was closed to make the chamber closed, obtaining the 1% hypoxic environment. Repeating the above air inflation procedure, after 2 min, the ventilation valve was closed, obtaining a 10% hypoxic environment. After 24 h of culture in hypoxia environment, the cells were taken out for lysis.

Statistical analysis

The quantitative data were expressed as mean ± standard deviation (SD). All the experiments were repeated for at least 3 times. The statistical analysis was conducted using a two-sided Student's t-test or one way ANOVA. P value < 0.05 was considered statistically significant.



Scheme S1. Schematic representation of the transmembrane current testing device. The solid-state nanochannel was fixed between two cells of the device. A pair of Ag/AgCl electrodes were placed into the two cells of device, respectively, and the electrolyte was 1 mM NaCl solution.

Table S1. The X-ray photoelectron spectroscopy (XPS) data of the GO membrane.

Name	Start BE	Peak BE	End BE	Height counts	At. %
C	297.98	286.32	279.18	55710.58	70.1
O	544.98	532.05	525.18	91227.04	29.9

Table S2. The XPS data of the GO-Crown membrane.

Name	Start BE	Peak BE	End BE	Height counts	At. %
C	297.98	284.91	279.18	41998.22	68.94
O	544.98	531.7	525.18	70363.88	27.2
N	409.98	398.75	392.18	5468.39	3.86

Table S3. The reported methods for detecting histone methyltransferase.

Method	Target (Histone methyltransferase)	Linear range	LOD	Reference
Functionalized two-dimensional nanochannels	G9a	20 fM-200 fM	21 fM	This study
A nanofluidic electric sensing device	G9a	ND	ND	[S1]
A DimerDye Disassembly Assay	PRDM9	ND	ND	[S2]
Fluorescent method based on arrayed supramolecular tandem assay	PRDM9	ND	ND	[S3]
Fluorescent assay based on magnetic separation	G9a	10 pM ~ 2 nM	12 pM	[S4]
AuNP-based colorimetric assay	SET 7/9 HMT	1 to 200 nM	0.2 nM	[S5]
A universal competitive fluorescence polarization activity assay	The methyltransferase catechol-O-methyltransferase (COMT)	ND	5 nM	[S6]
The enzyme-coupled continuous spectrophotometric assay	Protein arginine N-methyltransferase 1 (PRMT1)	ND	5 μM	[S7]
Host-assisted capillary electrophoresis	G9a	ND	ND	[S8]
A fluorescence-based supramolecular tandem assay	*Neurospora crassa* Dim-5 enzyme	ND	ND	[S9]
Continuous enzymatic assay	Neurospora crassa *Dim-5*	ND	ND	[S10]

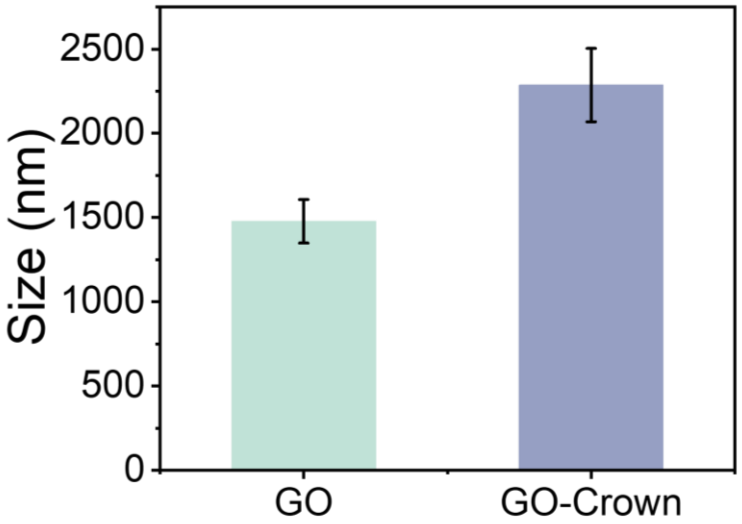


Fig. S1. The particle size of GO and GO-Crown dispersion.

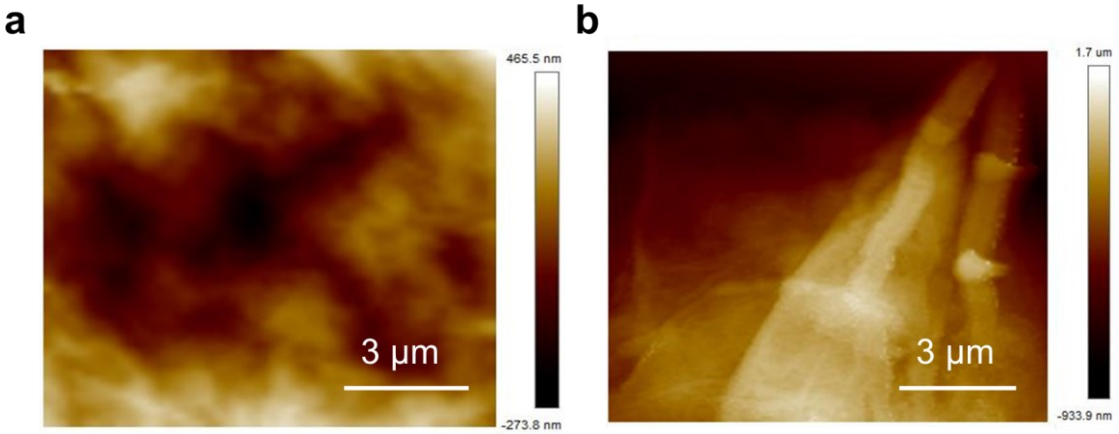


Fig. S2. The AFM images of (a) GO membrane and (b) GO-Crown membrane.

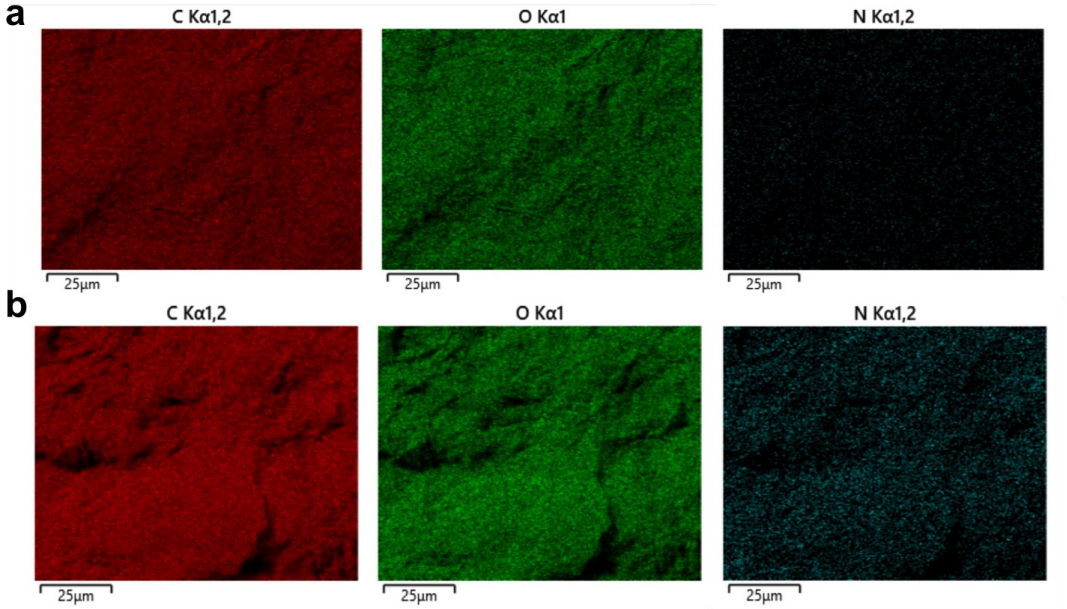


Fig. S3. Energy dispersive spectroscopy (EDS) images of (a) GO membrane and (b) GO-Crown membrane.

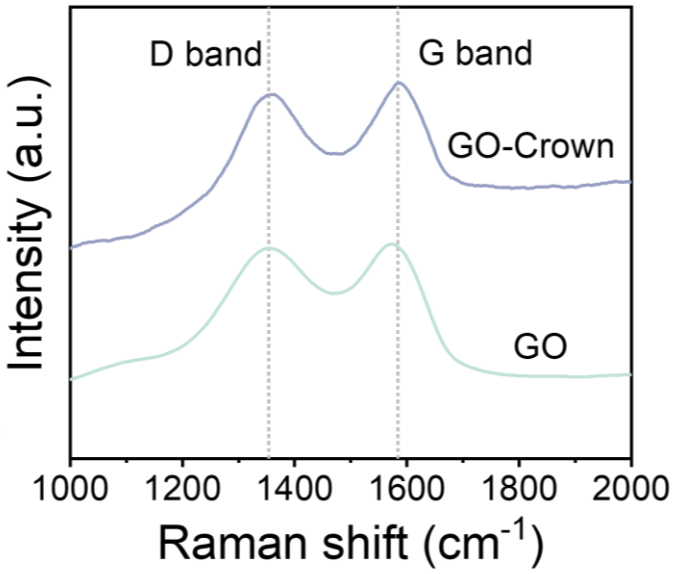


Fig. S4. The Raman spectra of GO-Crown membrane and GO membrane.

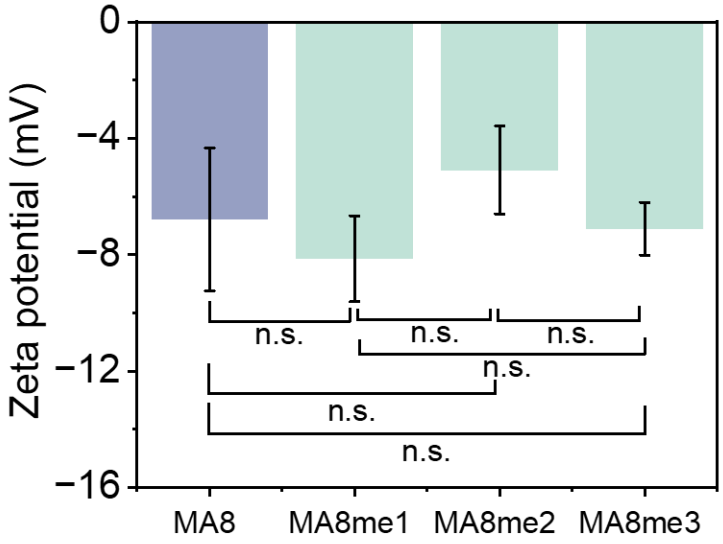
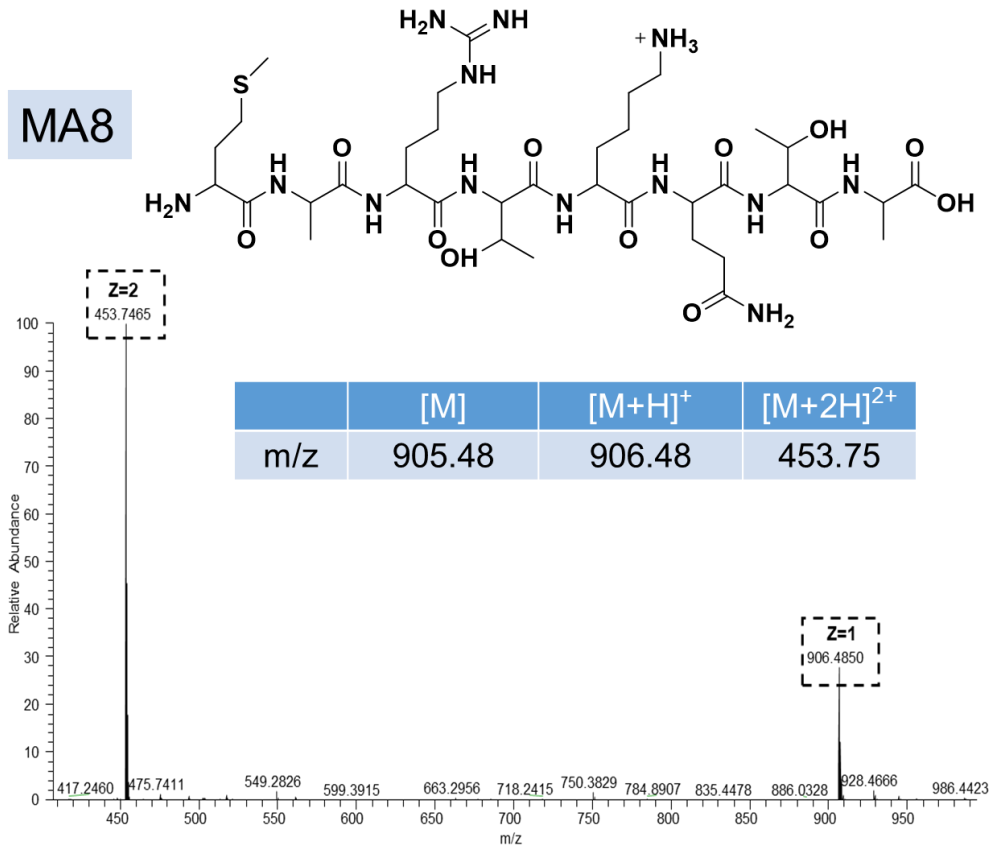
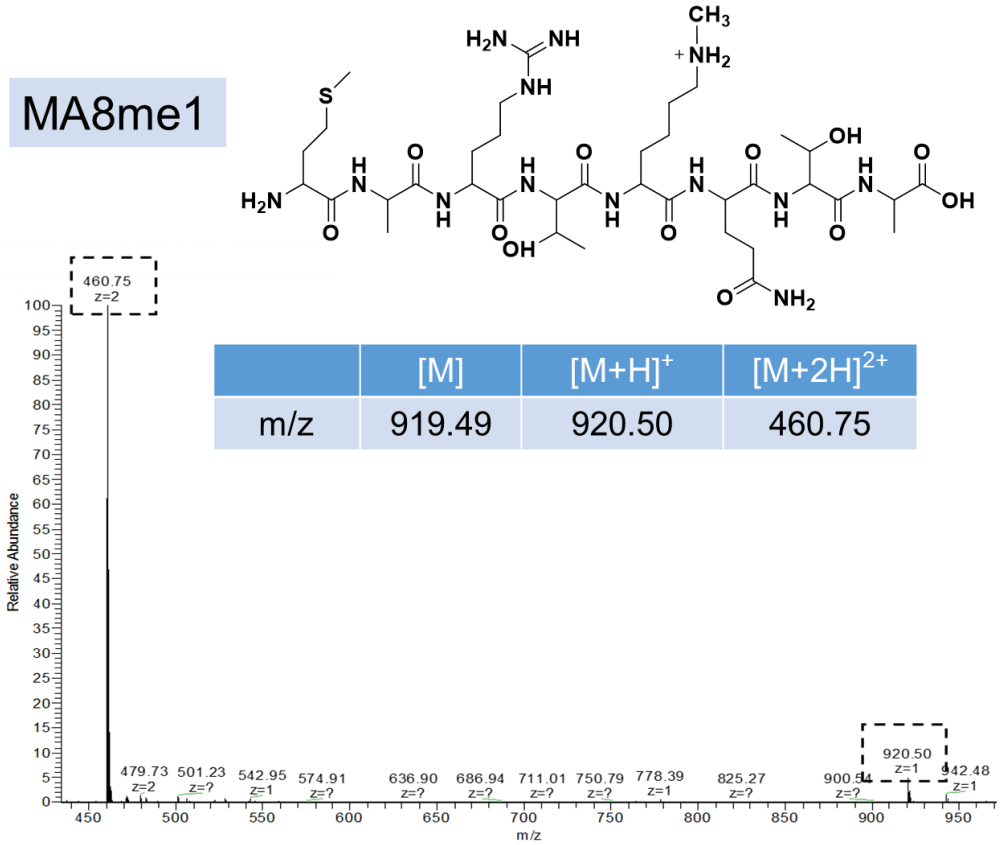


Fig. S5. The zeta potential of MA8, MA8me1, MA8me2 and MA8me3 peptides.



	[M]	$[M+H]^+$	$[M+2H]^{2+}$
m/z	905.48	906.48	453.75

Fig. S6. High-resolution mass spectrum (HR-MS) of MA8.



	[M]	$[M+H]^+$	$[M+2H]^{2+}$
m/z	919.49	920.50	460.75

Fig. S7. ESI-MS of MA8me1.

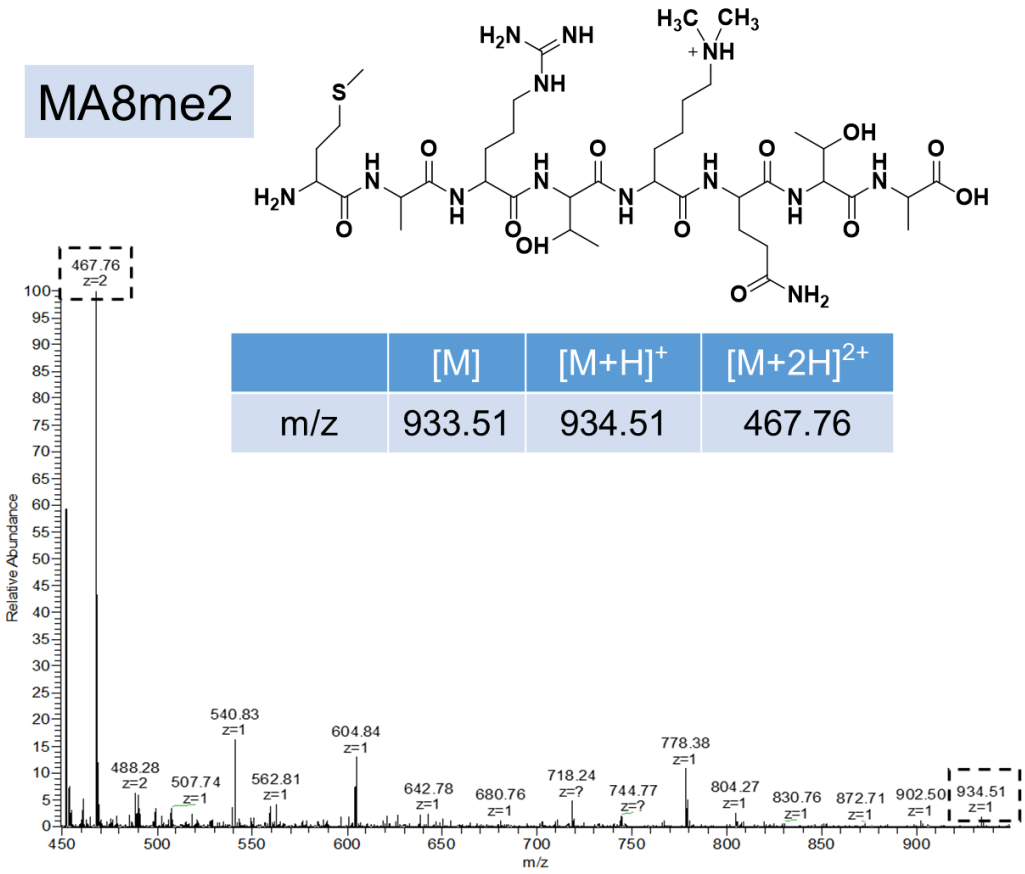


Fig. S8. EIS-MS of MA8me2.

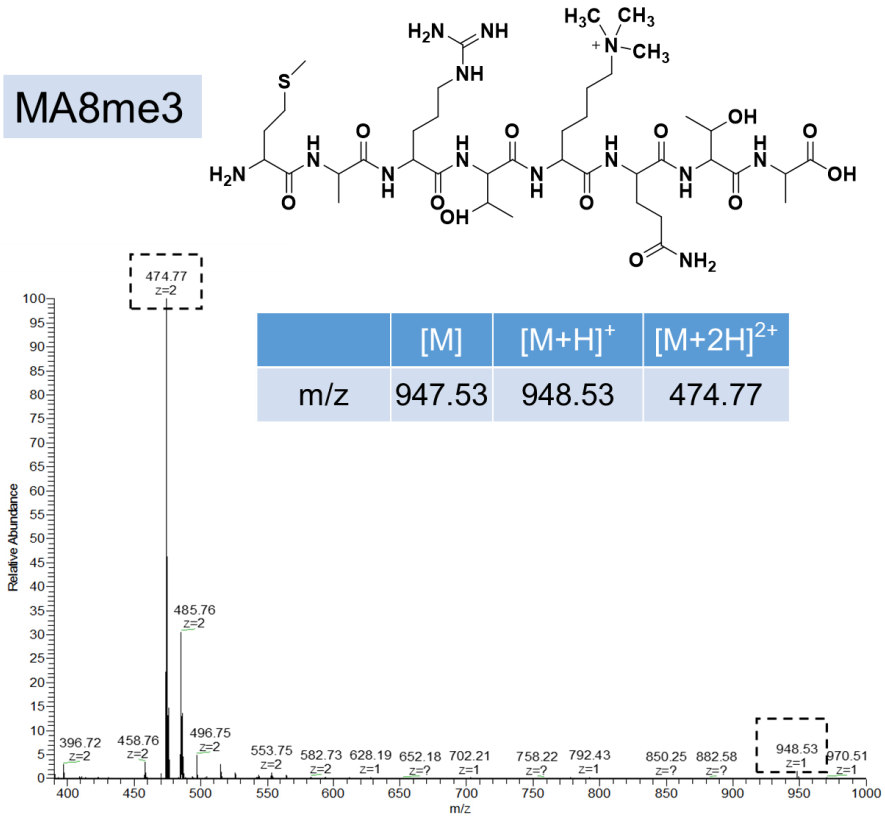


Fig. S9. EIS-MS of MA8me3.

Peptide	Kd (μM)
MA8	6.90±0.9
MA8me1	25.8±2.1
MA8me2	35.3±5.6
MA8me3	17.0±1.5

Fig. S10. The isothermal titration calorimetry (ITC) tests investigated the interaction of crown probe and peptides.

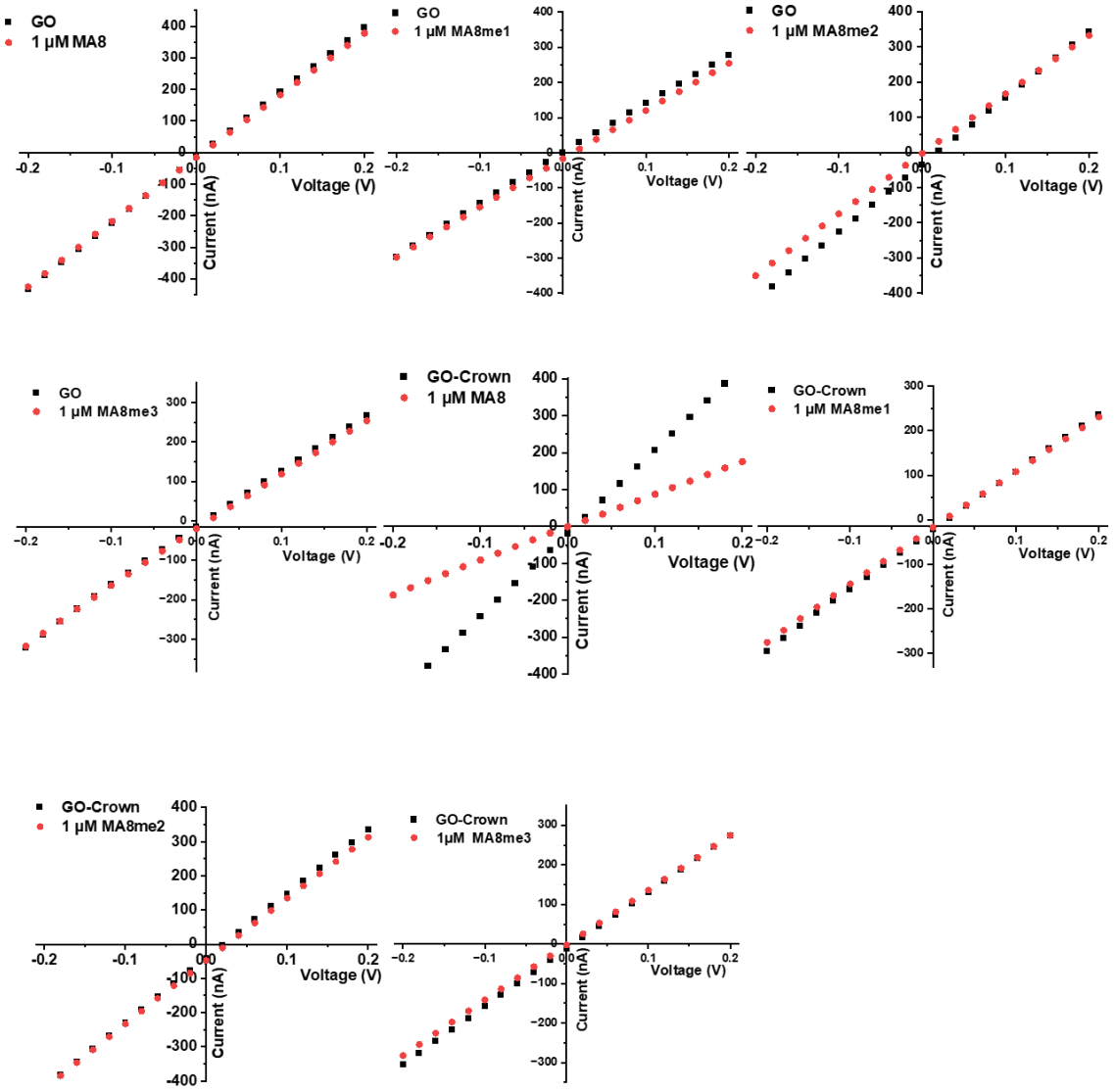


Fig. S11. I-V curves obtained at GO membrane and GO-Crown membrane before (black dashed line) and after (red dashed line) incubated with 1 μM peptides with different methylation degrees.

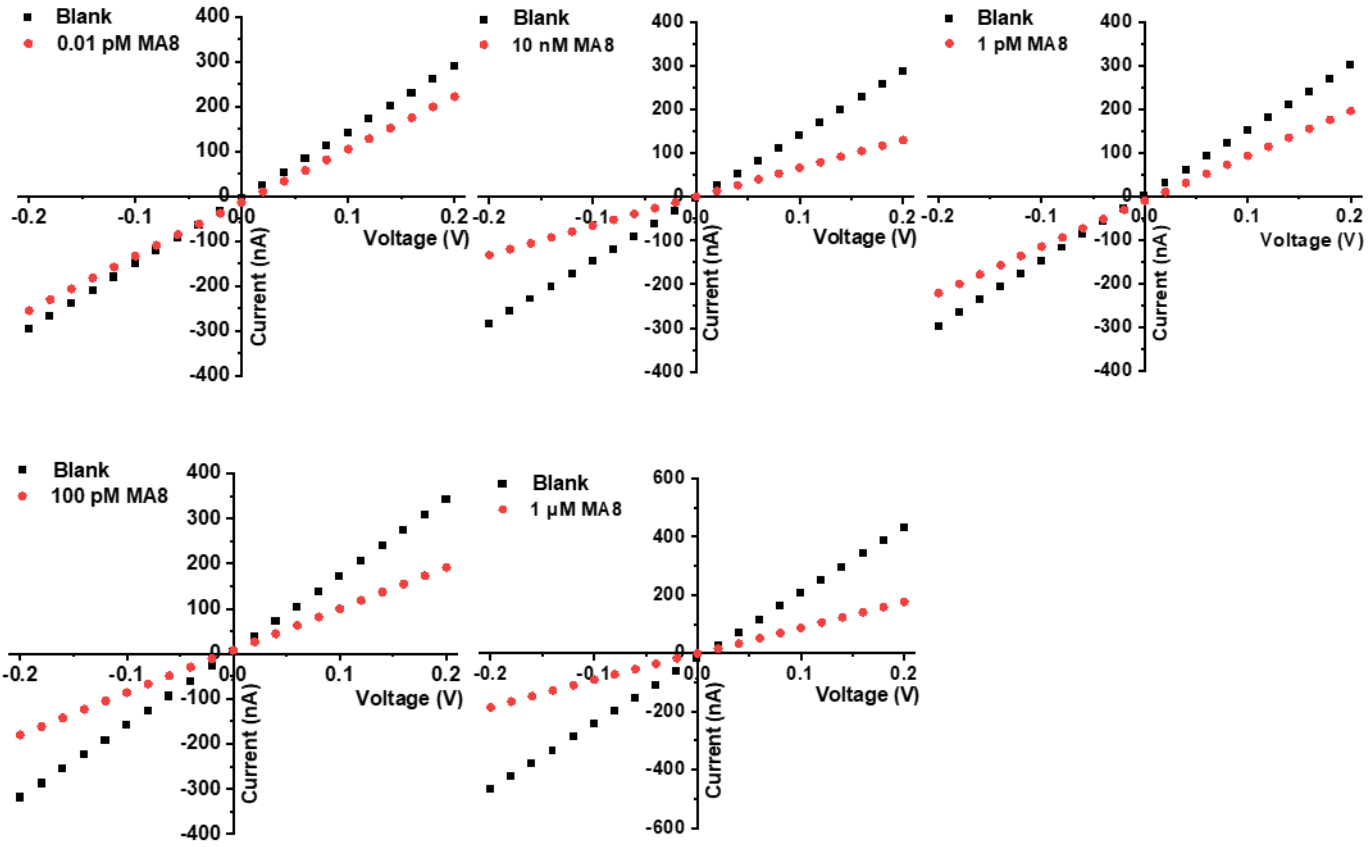


Fig. S12. I-V curves I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) incubated with different concentration of MA8 peptide.

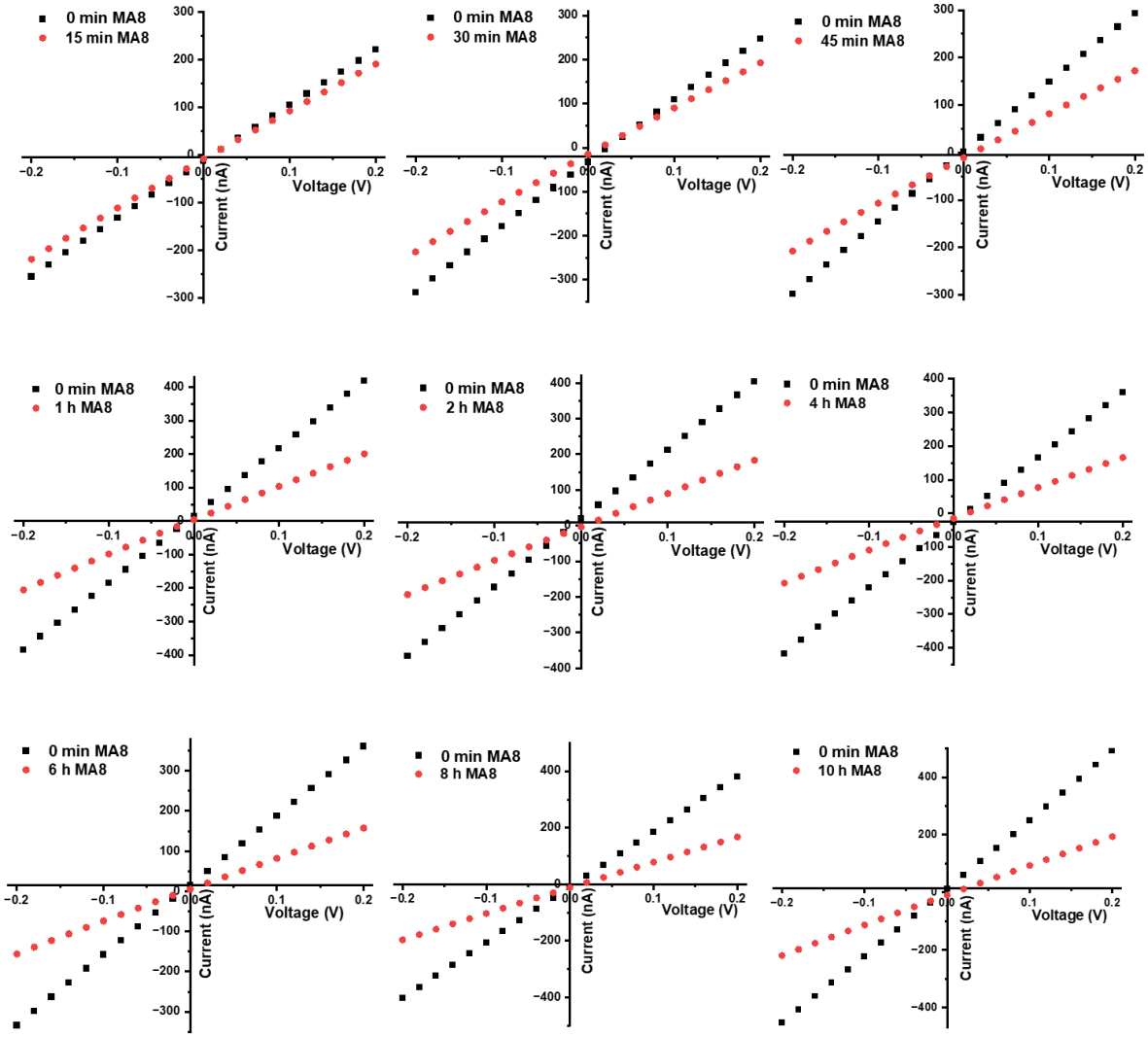


Fig. S13. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) different driving time (15 min-10 h).

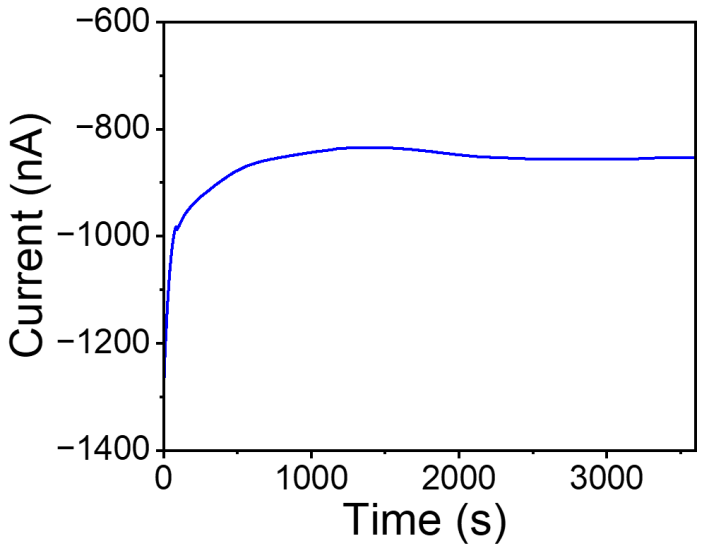


Fig. S14. The I-T image of MA8 peptides at GO-Crown membrane.

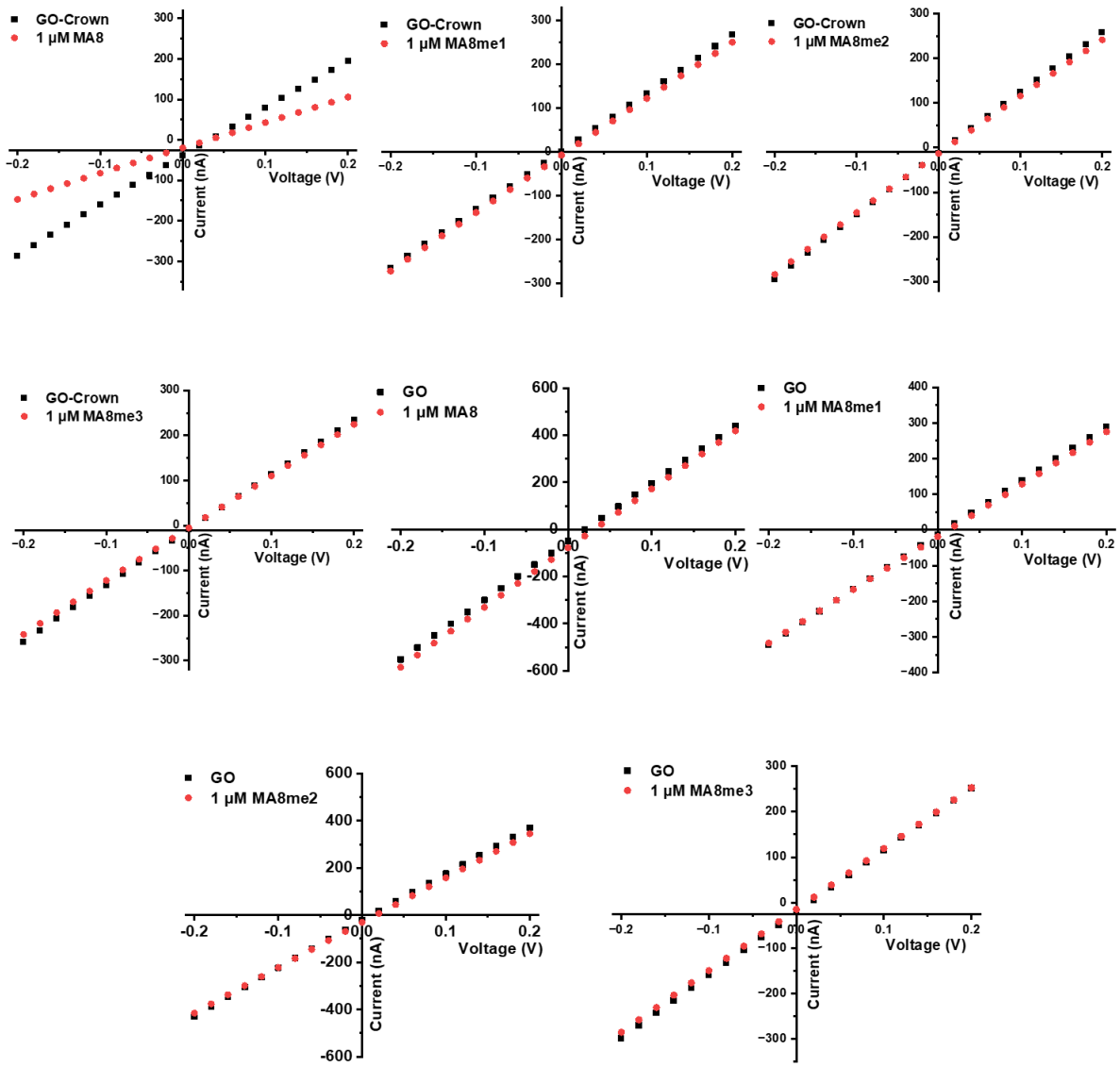


Fig. S15. I-V curves obtained at GO-Crown membrane and GO membrane before (black dashed line) and after (red dashed line) driving with 1 μM peptides with different methylation degrees.

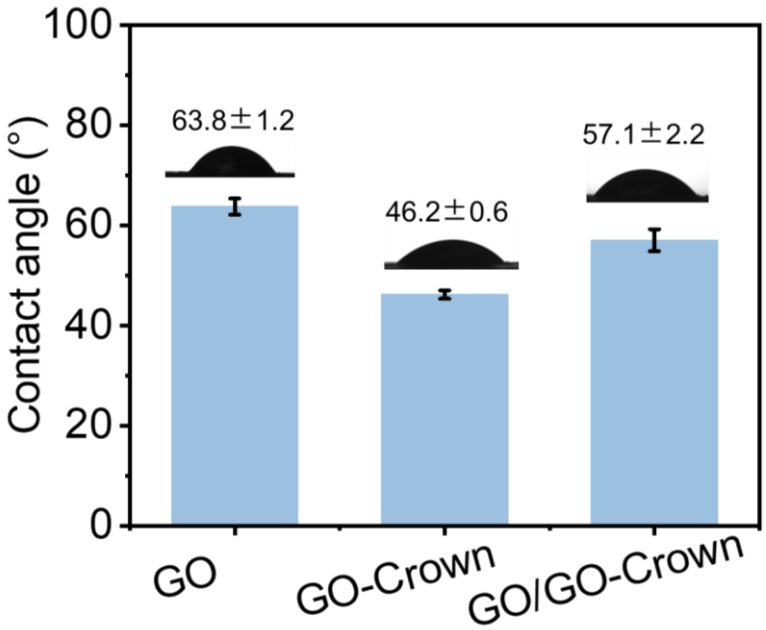


Fig. S16. The contact angle of GO membrane, GO-Crown membrane, and GO/GO-Crown membrane.

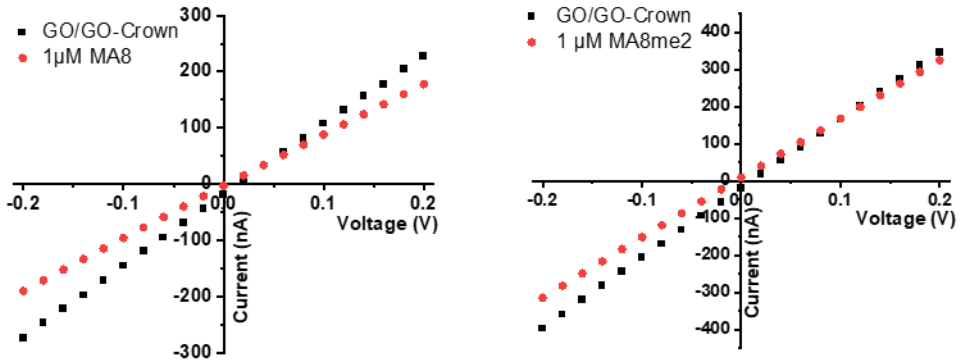


Fig. S17. Peptide-response curves at GO-Crown membrane, and GO/GO-Crown membrane.

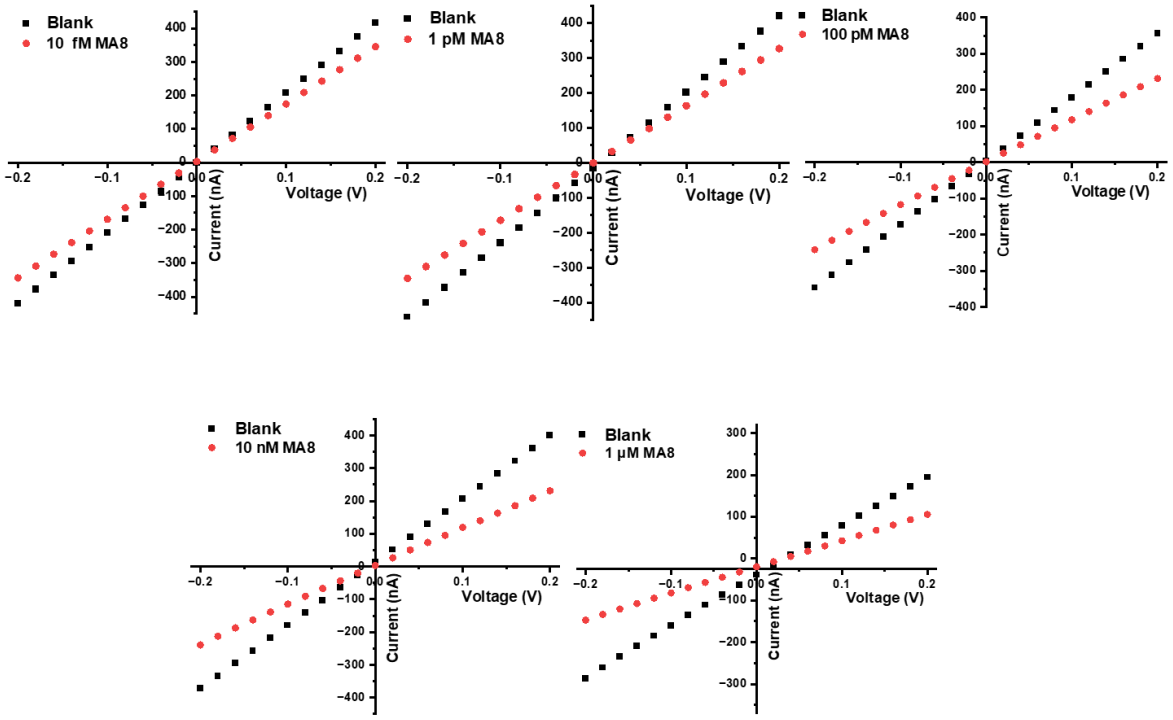


Fig. S18. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with MA8 peptides with different concentrations.

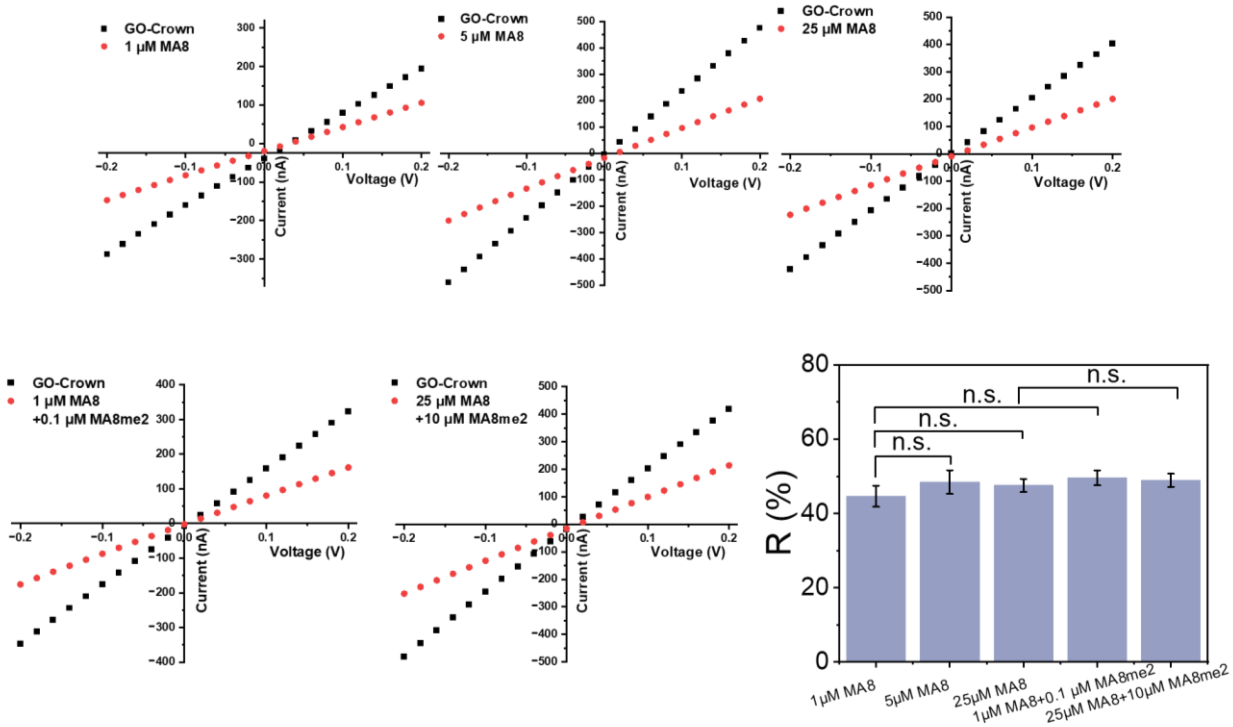


Fig. S19. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with high concentration peptides and mixed peptides.

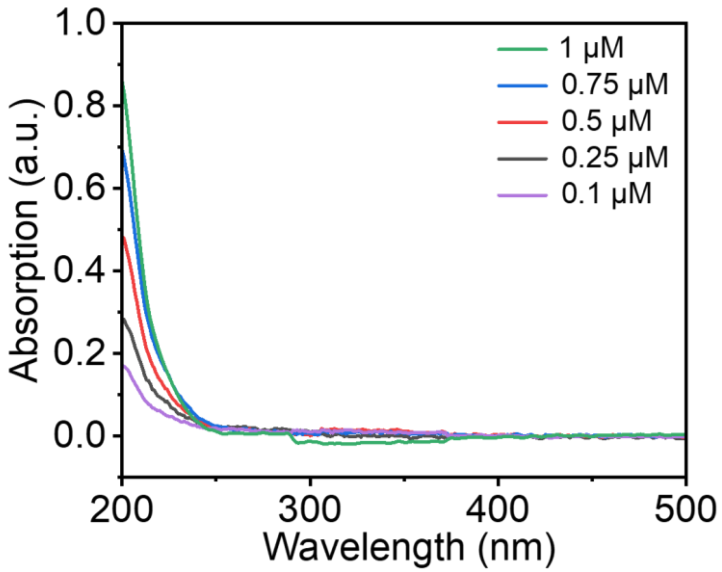


Fig. S20. The ultraviolet-visible (UV-vis) spectrophotometer of peptide with the concentration range from 0.1 μM -1 μM.

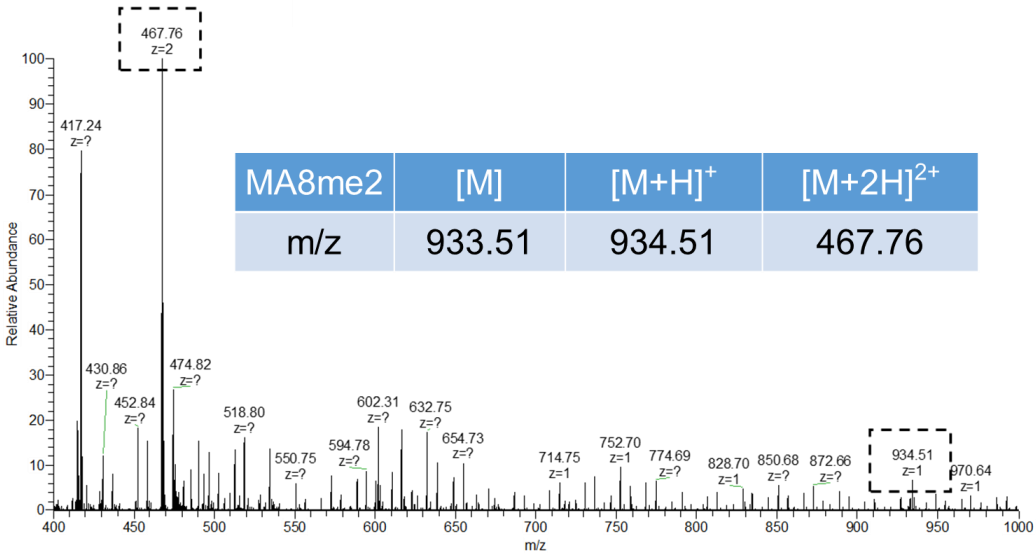


Fig. S21. ESI-MS of the solution in the receiving chamber after driving.

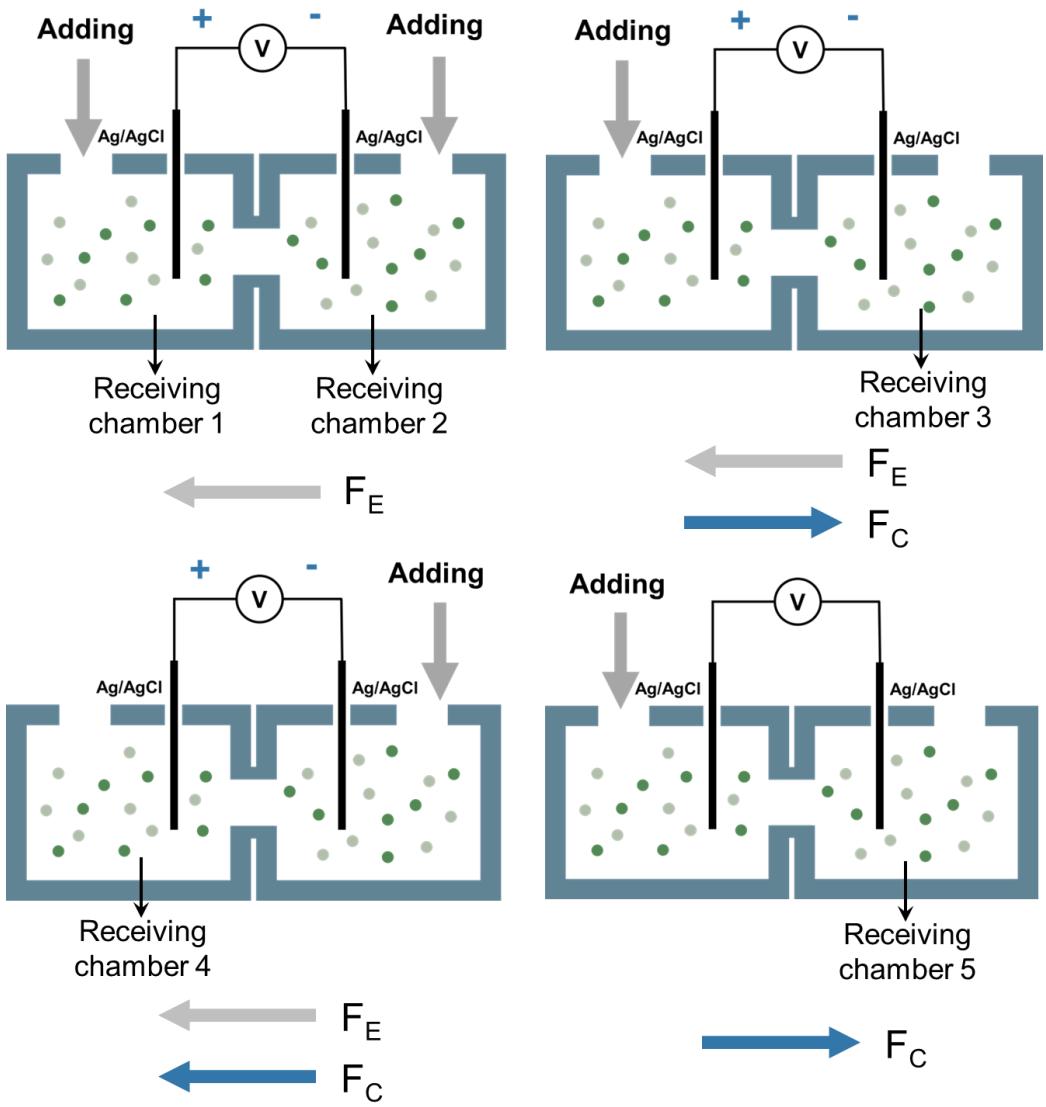


Fig. S22. The models to explore the influence of F_C (concentration difference) and F_E (electric field force) in the electric drive model.

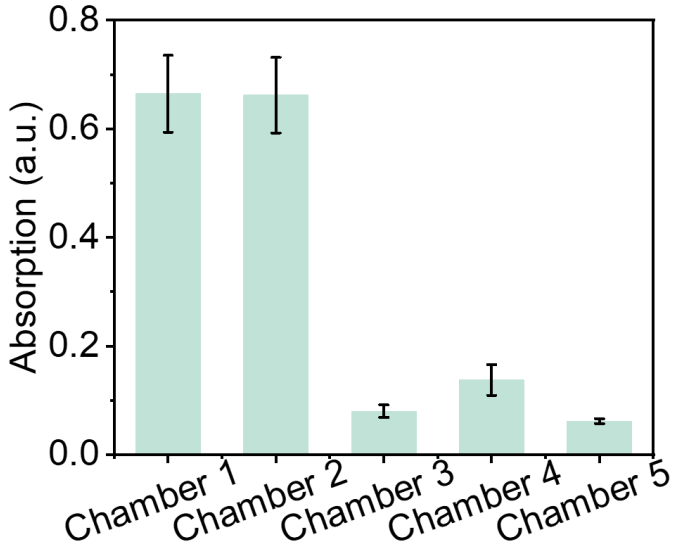


Fig. S23. The UV-vis absorption of five receiving chambers (abbreviated to chamber).

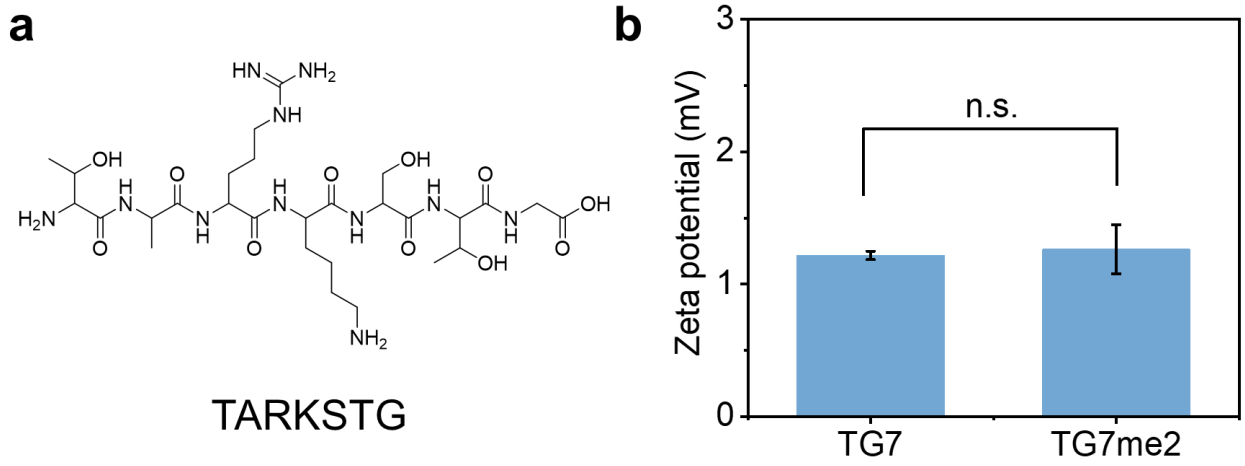


Fig. S24. (a) The chemical structures of TG7 peptide. (b) The zeta potential of TG7 and TG7me2 peptides.

m/z	[M]	$[M+H]^+$
TG7	719.40	720.40
TG7me1	734.04	735.04

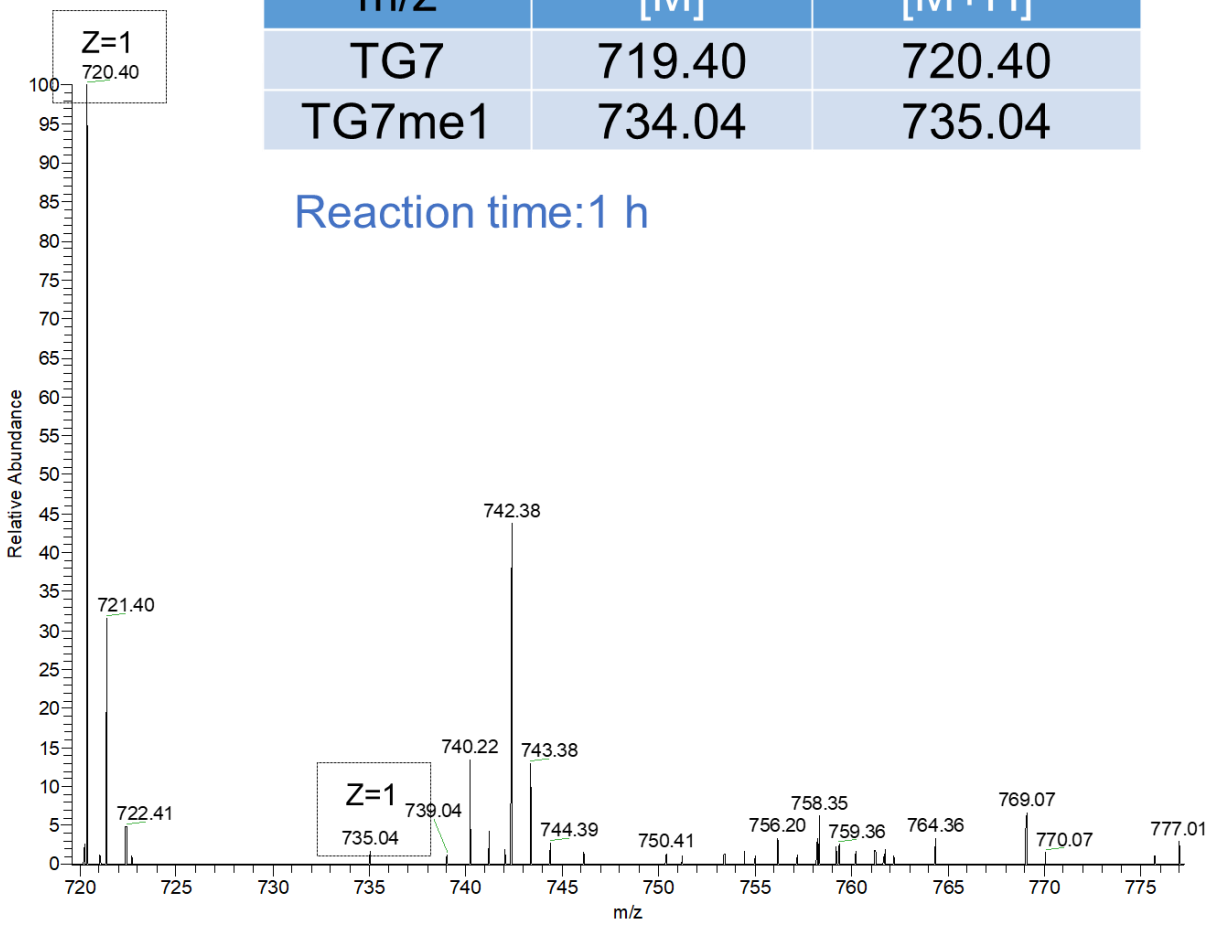


Fig. S25. ESI-MS of the reaction solution after enzymatic reaction of 1 h.

m/z	[M]	$[M+H]^+$
TG7	719.40	720.40
TG7me1	734.04	735.04
TG7me2	747.43	748.43

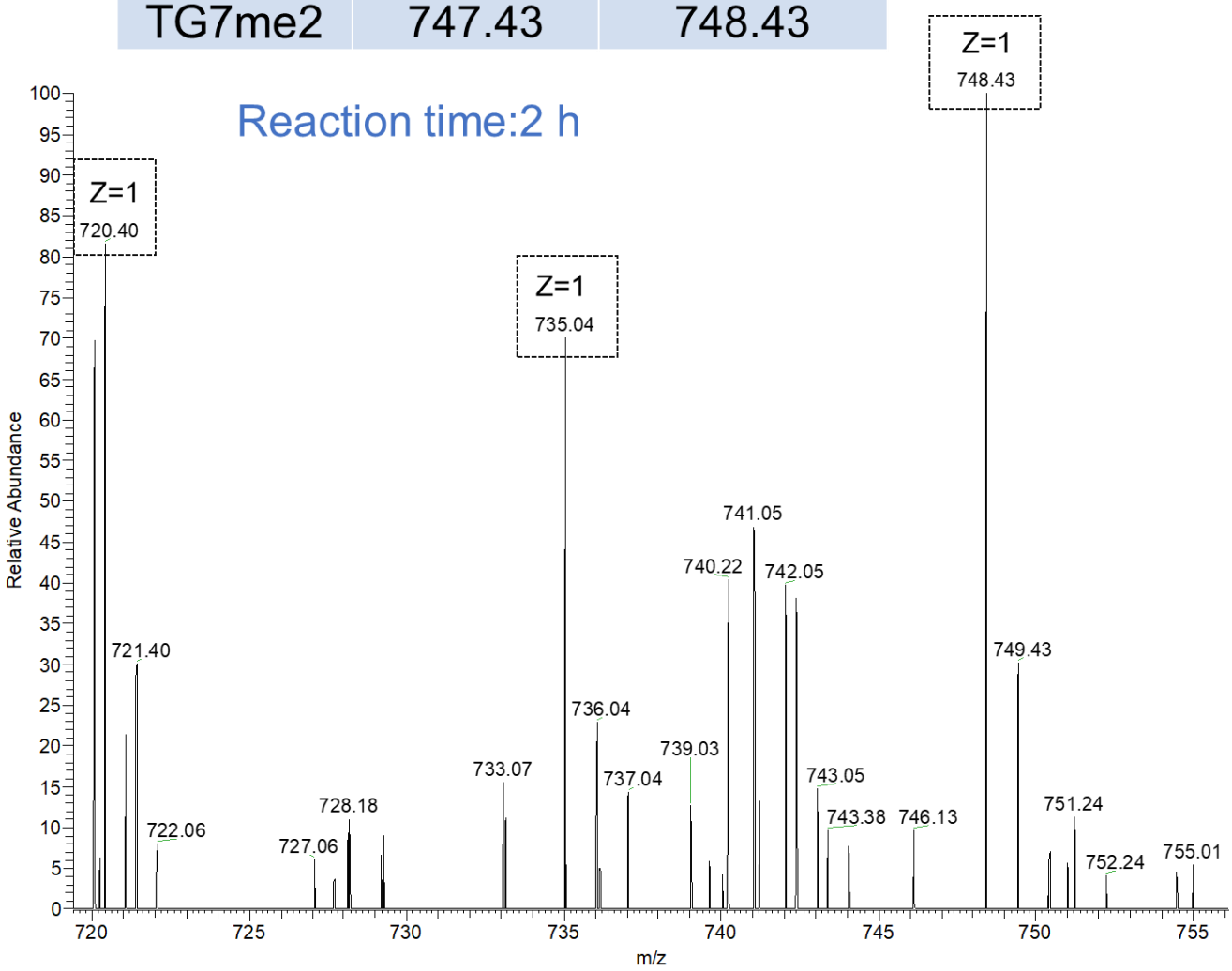


Fig. S26. ESI-MS of the reaction solution after enzymatic reaction of 2 h.

m/z	[M]	$[M+H]^+$
TG7	719.40	720.40
TG7me1	734.04	735.04
TG7me2	747.43	748.43

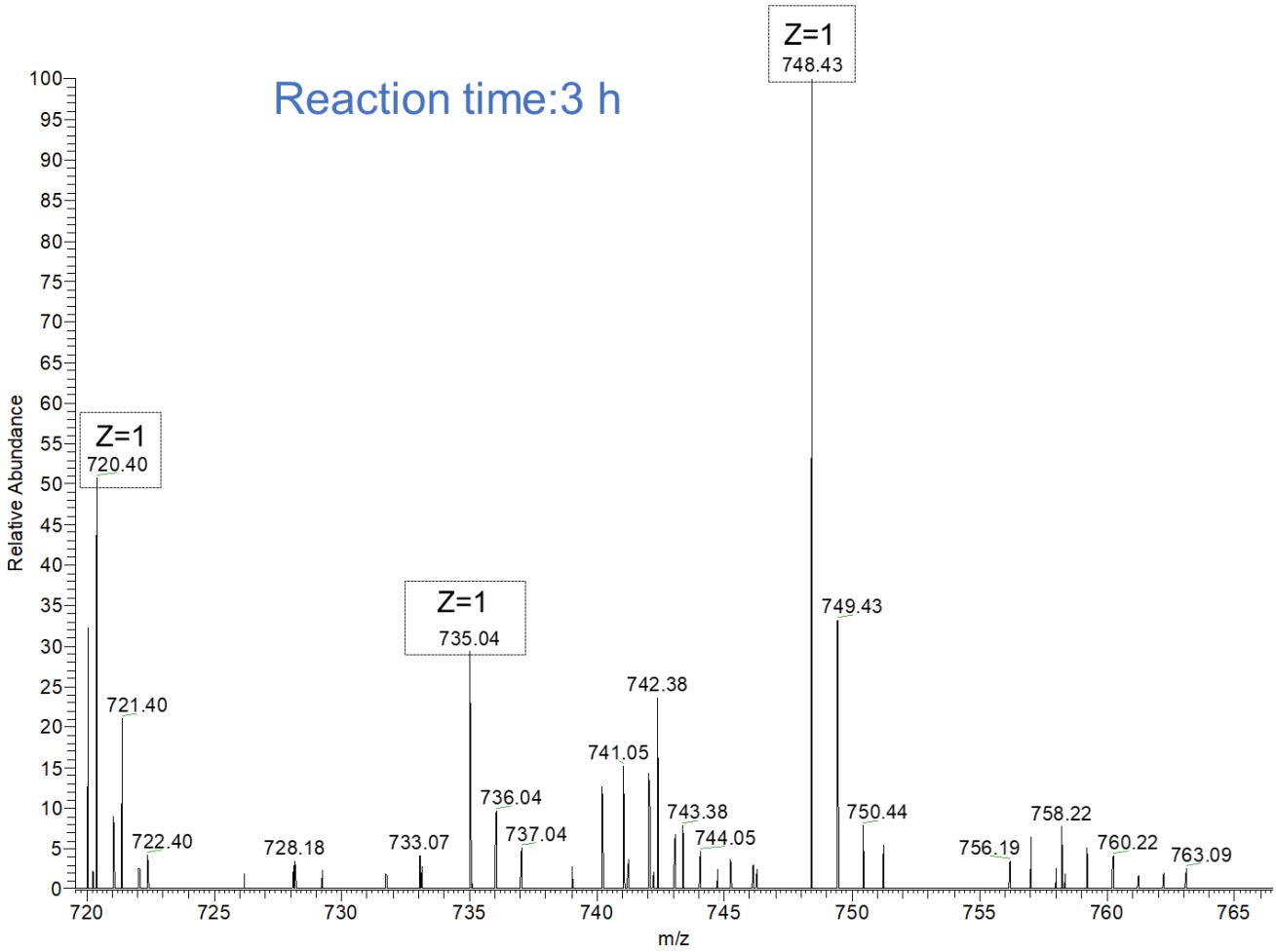


Fig. S27. ESI-MS of the reaction solution after enzymatic reaction of 3 h.

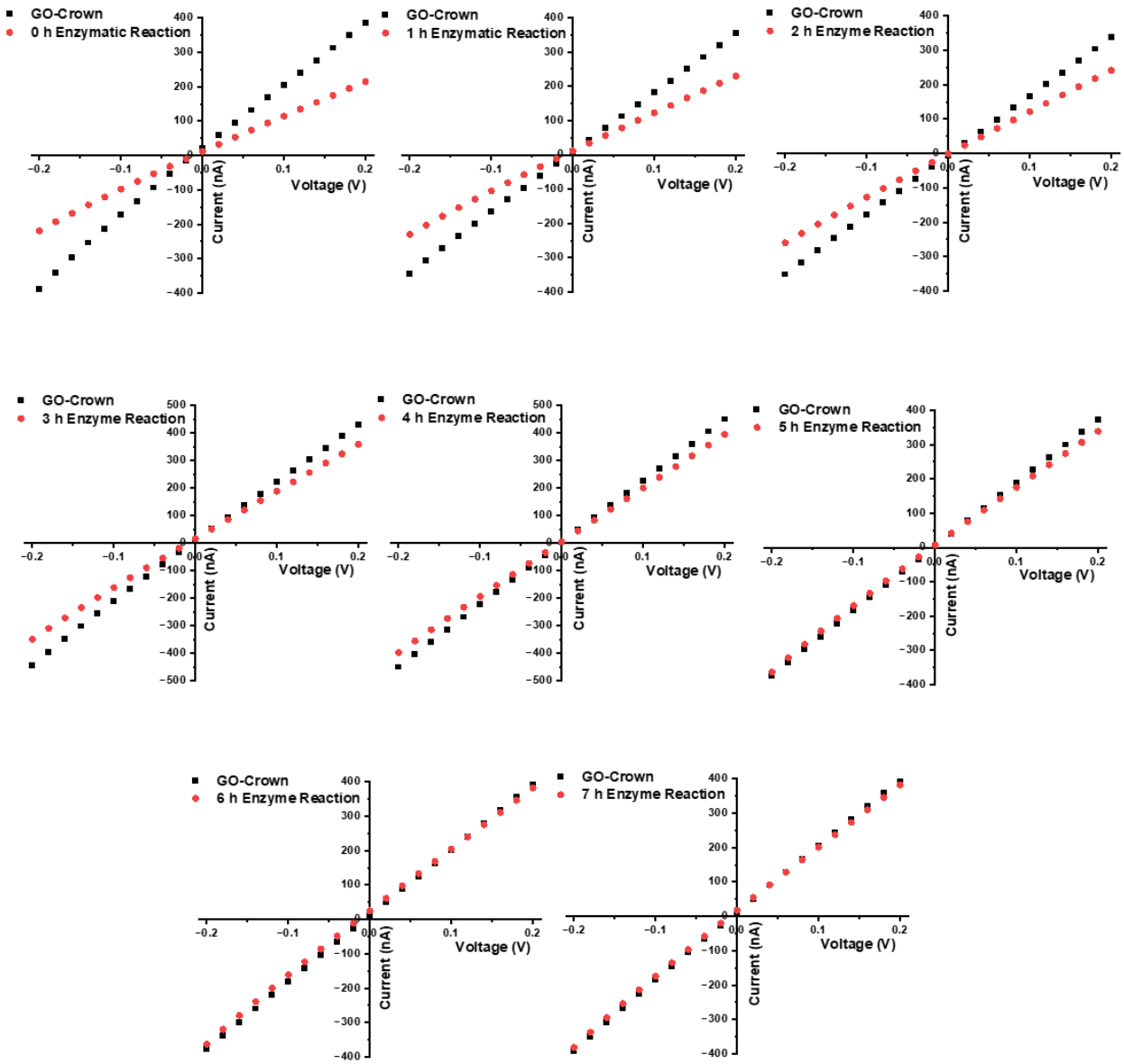


Fig. S28. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with enzyme reaction solution of different reaction times (0 h-7 h).

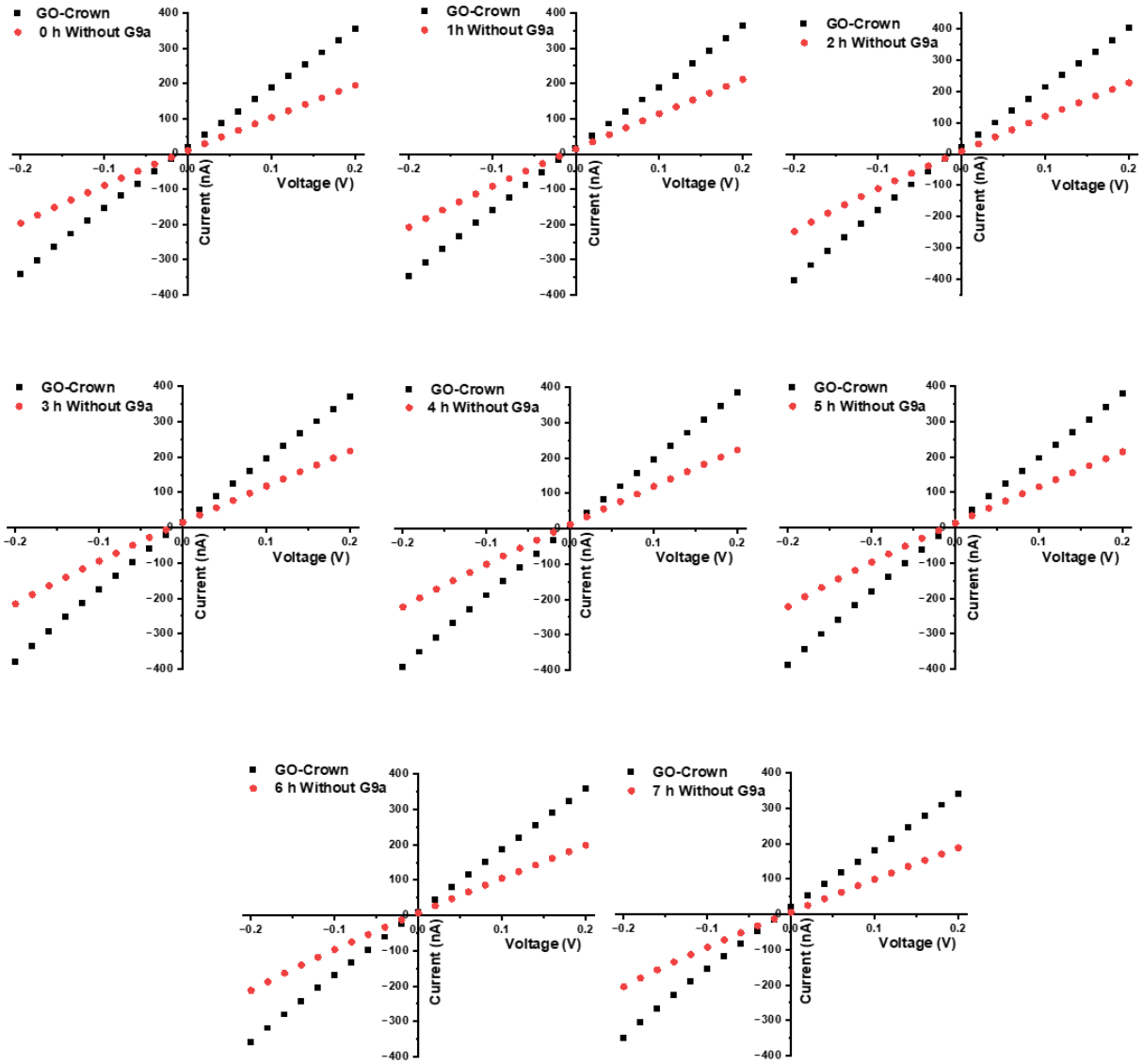


Fig. S29. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with enzyme-free mixture solution of different reaction time (0 h-7 h).

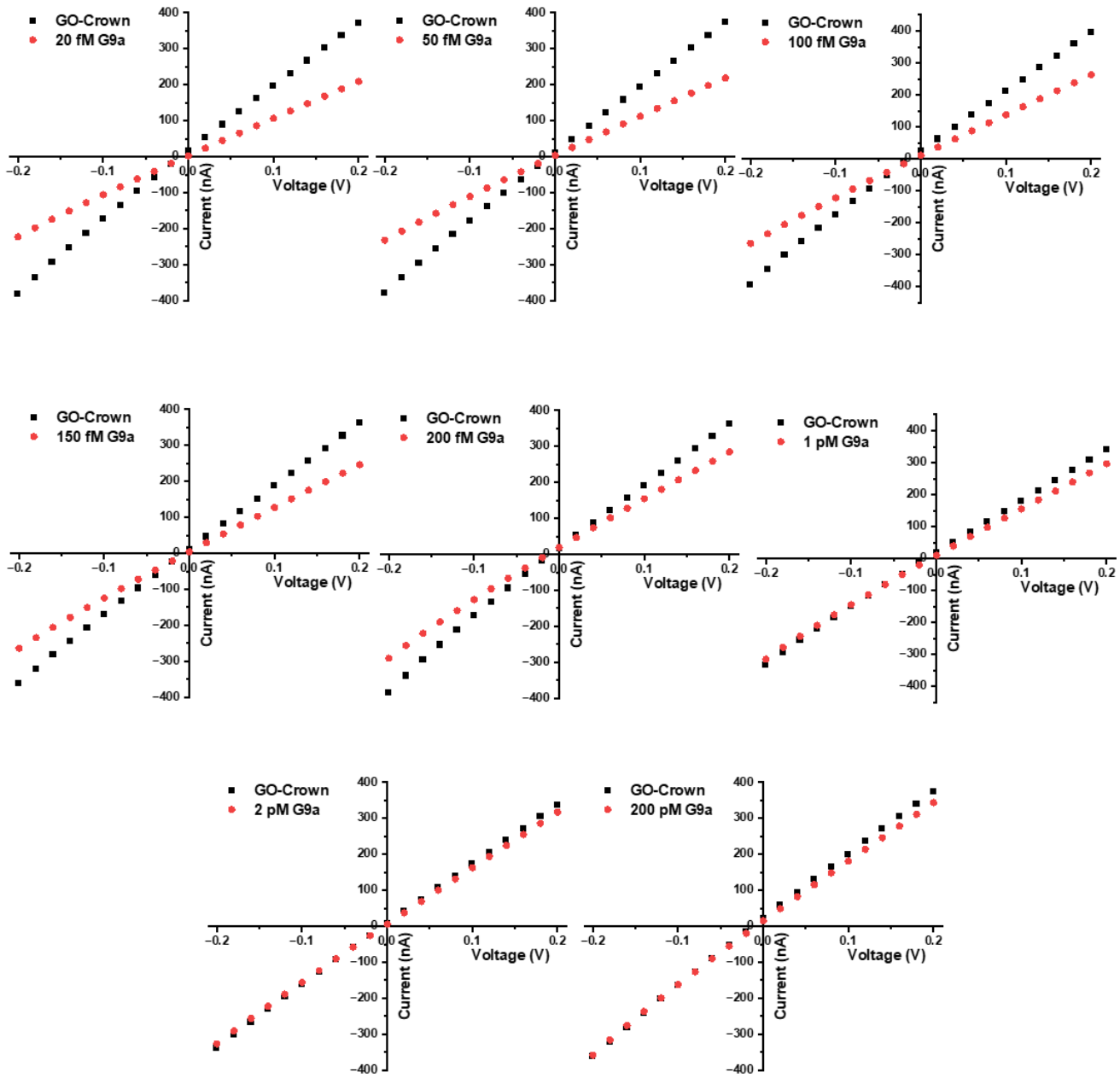


Fig. S30. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with the enzyme solution with differnet concentrations of G9a (20 fM-200 pM).

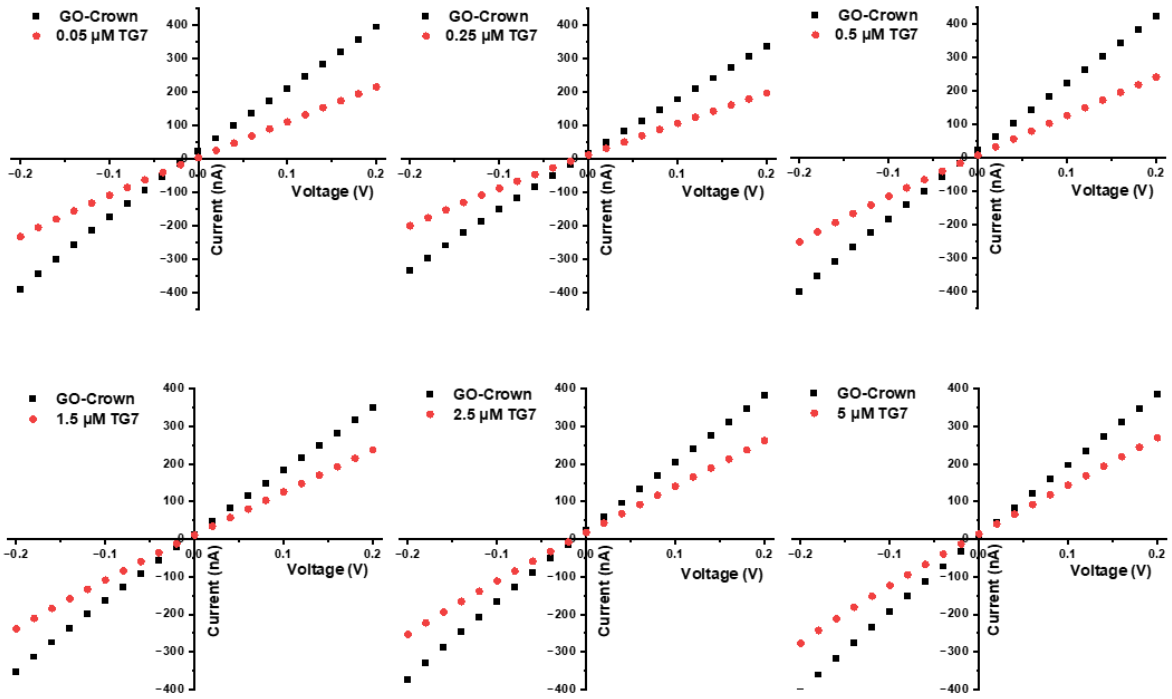


Fig. S31. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with different concentrations of substrate TG7 peptides (0.05 μM, 0.25 μM, 0.5 μM, 1.5 μM, 2.5 μM, and 5 μM). The temperature (25 ºC), enzyme concentration (20 nM) and reaction time (6 h) were controlled.

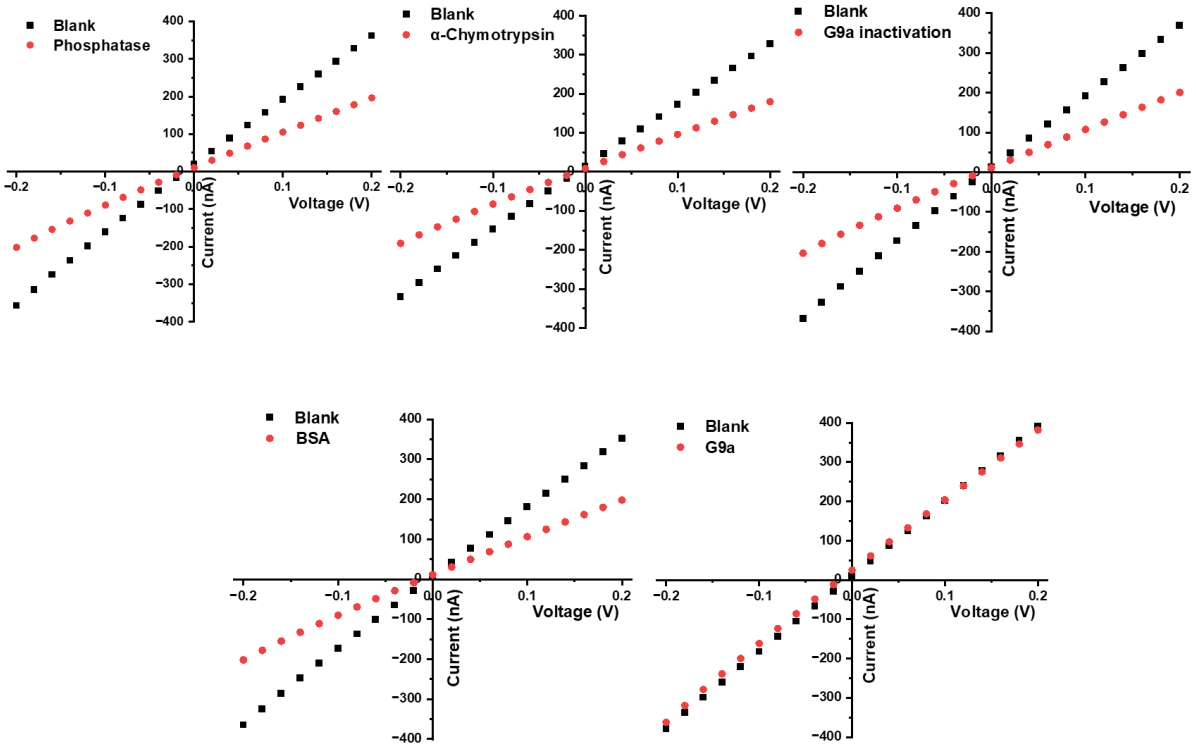


Fig. S32. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with α-chymotrypsin, phosphatase, bovine serum albumin (BSA), G9a inactivated (heated at 95 °C for 120 min) and G9a with the same concentration of 20 nM, respectively.

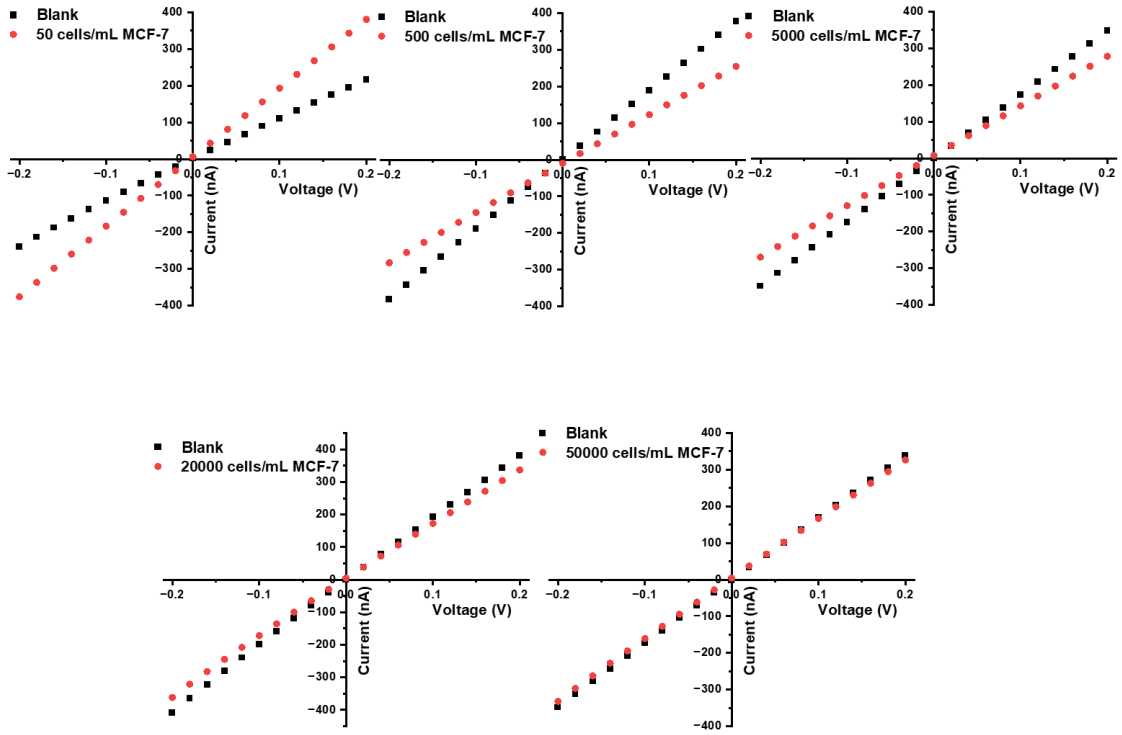


Fig. S33. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with cell lysates with the different number of MCF-7 cells (50 cells/mL-5000 cells/mL).

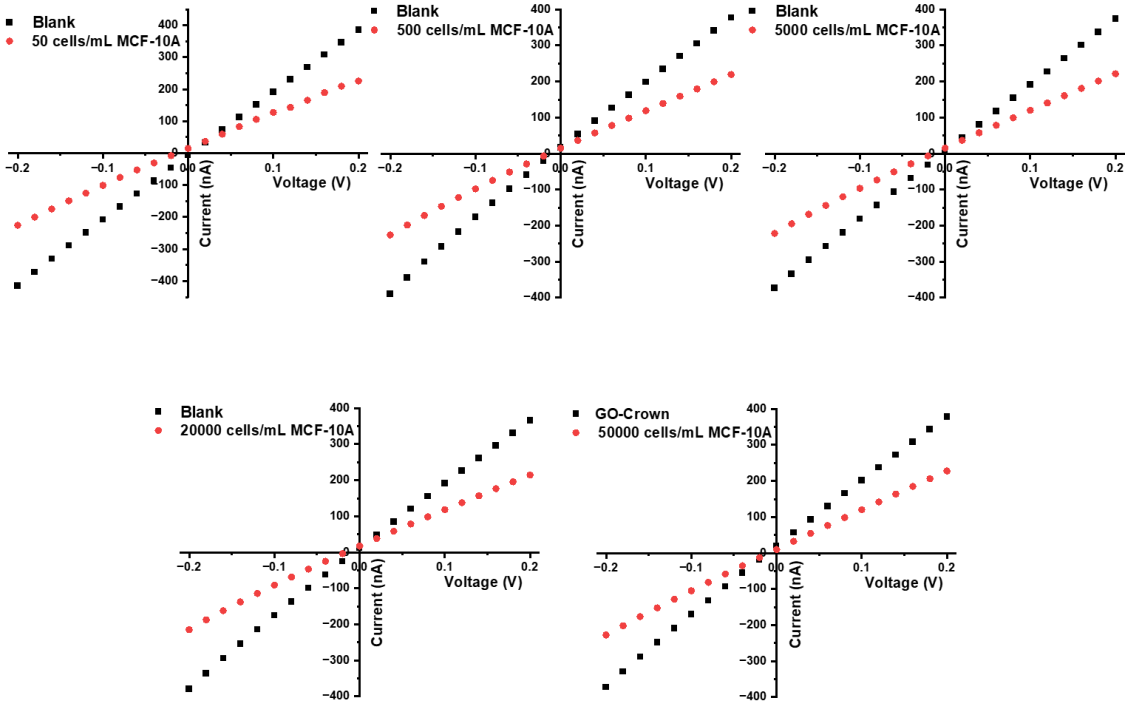


Fig. S34. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with cell lysates with the different number of MCF-10A cells (50 cells/mL-5000 cells/mL).

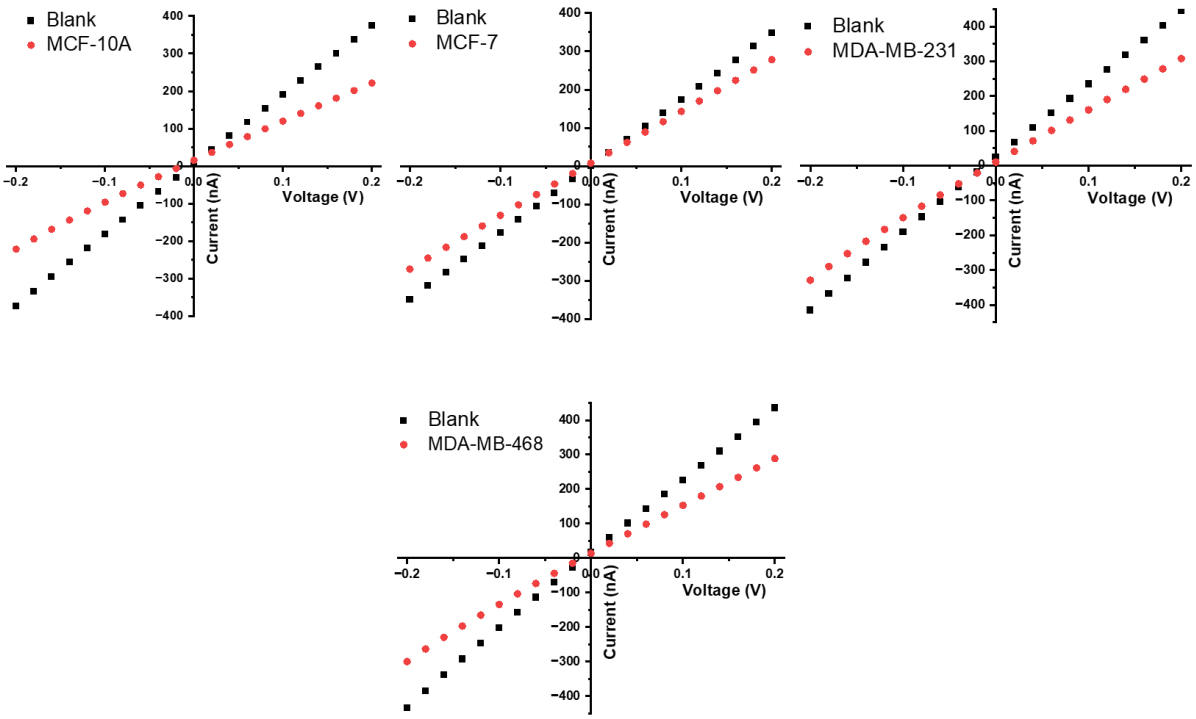
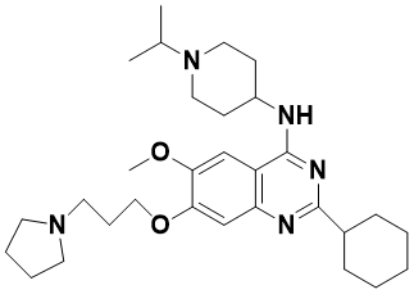


Fig. S35. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with cell lysates with the same number of cells (5000 cells/mL).



UNC0638

Fig. S36. The chemical structures of UNC0638 inhibitor.

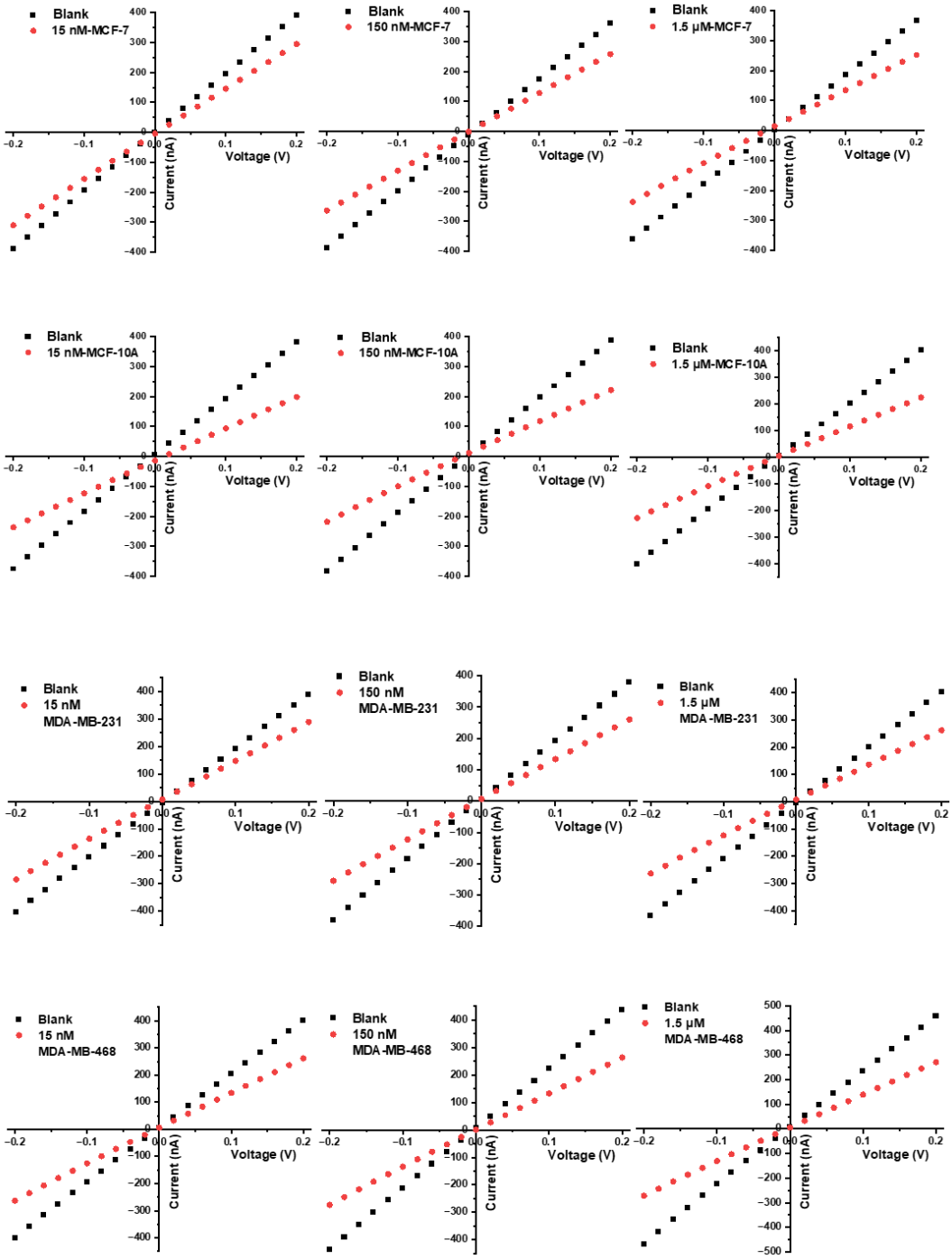


Fig. S37. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with various cell lysates treated with different concentration of UNC0638 inhibitor (15 nM, 150 nM, 1.5 µM).

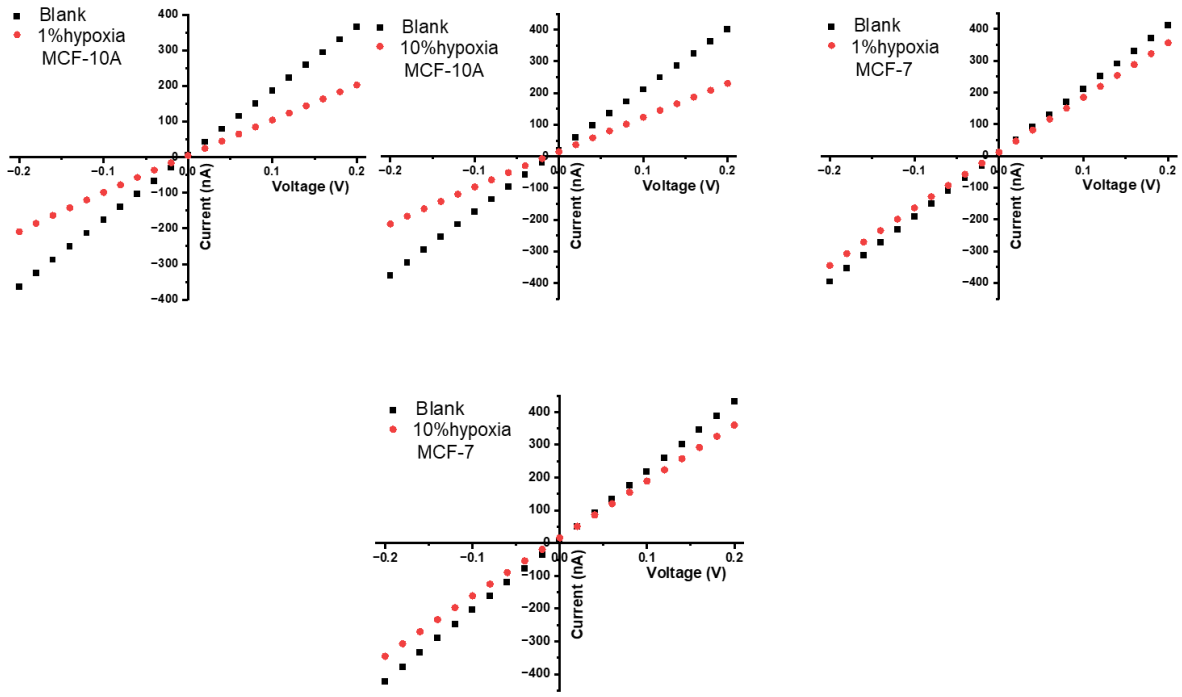


Fig. S38. I-V curves obtained at GO-Crown membrane before (black dashed line) and after (red dashed line) driving with various cell lysates (MCF-7, MCF-10A) treated with different concentrations of oxygen (1%, 10%).

References:

[S1] Xiong, Y.; Li, M.; Cao, Y.; Li, Z.; Chang, Y.; Zhao, X.; Qing, G. Nanofluidic device for detection of lysine methylpeptides and sensing of lysine methylation. *Anal. Chem.* **2023**, *95*, 7761-7769.

[S2] Beatty, M. A.; Borges-González, J.; Sinclair, N. J.; Pye, A. T.; Hof, F. Analyte-Driven Disassembly and Turn-On Fluorescent Sensing in Competitive Biological Media. *J. Am. Chem. Soc.* **2018**, *140*, 3500-3504.

[S3] Liu, Y.; Perez, L.; Gill, A. D.; Mettry, M.; Li, L.; Wang, Y.; Hooley, R. J.; Zhong, W. Site-selective sensing of histone methylation enzyme activity via an arrayed supramolecular tandem assay. *J. Am. Chem. Soc.* **2017**, *139*,10964-10967.

[S4] Ma, F.; Liu, M.; Wang, Z.; Zhang, C. Multiplex detection of histone-modifying enzymes by total internal reflection fluorescence-based single-molecule detection. *Chem Commun.* **2016**, *52*, 1218-1221.

[S5] Zhen, Z.; Tang, L. J.; Long, H.; Jiang, J. H. Enzymatic immuno-assembly of gold nanoparticles for visualized activity screening of histone-modifying enzymes. *Anal. Chem.* **2012**, *84*, 3614-3620.

[S6] Graves, T. L.; Zhang, Y.; Scott, J. E. A universal competitive fluorescence polarization activity assay for S-adenosylmethionine utilizing methyltransferases. *Anal. Biochem.* **2008**, *373*, 296-306.

[S7] Dorgan, K. M.; Wooderchak, W. L.; Wynn, D. P.; Karschner, E. L.; Alfaro, J. F.; Cui, Y.; Zhou, Z. S.; Hevel, J. M. An enzyme-coupled continuous spectrophotometric assay for S-adenosylmethionine-dependent methyltransferases. *Anal. Biochem.* **2006**, *350*, 249-255.

[S8] Lee, J.; Chen, J.; Sarkar, P.; Xue, M.; Hooley, R. J.; Zhong, W. Monitoring the crosstalk between methylation and phosphorylation on histone peptides with host-assisted capillary electrophoresis. *Anal. Bioanal. Chem.* **2020**, *412*, 6189-6198.

[S9] Florea, M.; Kudithipudi, S.; Rei, A.; Gonz á lez-Álvarez, M. J.; Jeltsch, A.; Nau, W. M. A Fluorescence-Based Supramolecular Tandem Assay for Monitoring Lysine Methyltransferase Activity in Homogeneous Solution. *Chem. Eur. J.* **2012**, *18*, 3521-3528.

[S10] Rathert, P.; Cheng, X.; Jeltsch, A. Continuous enzymatic assay for histone lysine methyltransferases. *BioTechniques* **2007**, *43*, 602-608.