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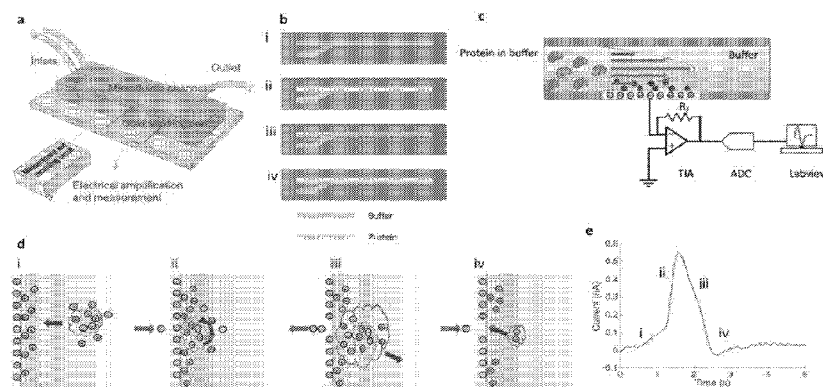


FIG. 1

(57) Abstract: Methods, systems, and devices are disclosed for studying physical or chemical properties of molecules, and/or interactions between molecules such as protein-ligand interactions. The methods, systems, and devices involve transient induced molecular electronic spectroscopy (TIMES). In some configuration, a microfluidic channel having at least one inlet and at least one outlet is used for holding molecules for analyzing the molecules or interactions between molecules.

**TRANSIENT INDUCED MOLECULAR ELECTRONIC SPECTROSCOPY
METHOD FOR STUDY OF MOLECULE INTERACTIONS**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent document claims benefit and priority of U.S. Provisional Patent
5 Application No. 62/305,766 entitled “TRANSIENT INDUCED MOLECULAR
ELECTRONIC SPECTROSCOPY (TIMES) METHOD FOR STUDY OF PROTEIN-
LIGAND INTERACTIONS,” filed on March 9, 2016. The entire content of the
aforementioned patent application is incorporated by reference as part of the disclosure of
this patent document.

**STATEMENT REGARDING FEDERALLY
SPONSORED RESEARCH OR DEVELOPMENT**

[0002] This invention was made with government support under Grant Number
NSF1610516, awarded by National Science Foundation. The government has certain rights
in the invention.

TECHNICAL FIELD

[0003] This patent document relates to systems, devices, and processes for detecting
interactions between molecules, e.g., protein-ligand interactions.

BACKGROUND

[0004] Protein-ligand interaction plays the central role in biomedical process and drug
20 discovery due to its scientific significance and practical applications. While computer
simulations and high-throughput screening methods have been widely applied to perform
early stage screening of drug candidates, limited methods are available to investigate the
effect of interactions between molecules such as protein-ligand interaction without any
external disruptions. This patent document provides techniques that, among other benefits,
25 overcome these limitations in the field.

SUMMARY

[0005] In one aspect, this document relates to a device for measuring a transient induced
molecular electronic spectroscopy (TIMES) signal of a molecule.

[0006] In one example aspect, a device for measuring a transient induced molecular

electronic spectroscopy (TIMES) signal of a molecule is disclosed. The device includes a microfluidic channel, a sensing pad comprising an electrode, a low-noise amplifier circuit, at least one inlet coupled to a first end of the microfluidic channel, and at least one outlet coupled to a second end of the microfluidic channel. The electrodes are connected to the amplifier circuit.

[0007] In another example aspect, a method for detecting binding of molecules including receiving a signal generated by the dipole moment change when a first molecule and a second molecule forms a complex, performing analog-to-digital conversion of the signal to generate a digitized signal, processing the digitized signal by a processor to detect binding between the first molecule and the second molecule. In some embodiments, the first and second molecules are a protein and a ligand, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] FIGs 1(a)-1(e) show experimental setup and illustration of the physical process that gives rise to the TIMES signal. (a) 3D view of the TIMES setup consisting of a polydimethylsiloxane (PDMS)-based microfluidic channel with gold electrodes and a low-noise transimpedance amplifier circuit. (b) The top view of microfluidic channel and the experimental work flow for channel conditioning, protein loading, and testing. (b,i) Before the experiment, the channel was flushed and filled with buffer injected from both inlets. (b,ii) One of the inlets was replaced with protein (or other molecules of interest) laden buffer to fill the channel. (b,iii) The syringe pump driving protein flow was stopped and the other syringe pump driving the buffer was turned on to wash the channel. This procedure completed the conditioning of the system before test, leaving a liquid interface between protein and buffer solutions near the input of the channel. (b,iv) To start the experiment, the flow of the buffer was stopped and the protein laden solution flowed through microfluidic channel. (c) Side view schematic of laminar flow in the microfluidic channel, charge distribution at the electrode/liquid interface, and the measurement and recording circuit system. (d) Illustration of charge movement, protein reorientation, and signal generation. (d,i) Local charge neutrality was maintained while a molecule was diffused towards the gold electrode. (d,ii) Ion redistribution and protein dipole moment reorientation occurred when the protein was near the gold electrode. Charge was transferred between the gold electrode and the external circuit due to the image charge effect. (d,iii) Protein left the electrode and dragged the local ions via electrostatic interactions. (d,iv) Ions returned to the electrode and the steady state was restored. (e) The measured TIMES signal produced by 500 nM thermolysin.

[0009] FIGs 2(a)-2(c) show induced charge response $q(t)$, and the adsorption time τ_s , of a single protein or protein/ligand complex under different flow rates (shear stress) obtained from the TIMES signal using Eq. (2). Induced charge response by thermolysin (a) and thermolysin/phosphoramidon complex (b) under different flow rate (shear stress) from 30 $\mu\text{L}/\text{min}$ (6.67 Pa) to 450 $\mu\text{L}/\text{min}$ (100 Pa). (c) Shear stress dependent surface adsorption time of thermolysin and thermolysin /phosphoramidon complex.

[0010] FIGs 3(a)-3(f) show TIMES signals with different ratios of protein ligand concentrations. 5 mM Tris-HCl buffer was used for all the experiments. Solutions with different concentrations of protein and ligand were mixed under room temperature for 3 hours before running the experiment. (a) Signals from 100 μM trypsin mixed with 0, 50, 100, and 200 μM of *p*-ABA. (b) The calculated equilibrium dissociation constant K_D from TIMES signals using Eq. (3). (c) Histogram of K_D for trypsin and *p*-ABA reaction from 1000 data points over the 1s measurement period. The mean value of K_D is also shown in the figure. (d) Signals from 300 nM thermolysin mixed with 0, 150, 300, and 600 nM of phosphoramidon. (e) The calculated equilibrium dissociation constant K_D from TIMES signals using Eq. (3). (f) Histogram of K_D for thermolysin and phosphoramidon reaction from 1000 data points over the 1s measurement period. The mean value of K_D is also shown in the figure.

[0011] FIGs 4(a)-4(b) show control experiments. (a) Signal showing the opposite polarity to the signal in Fig. 2d with a reversed protein concentration gradient. To reverse the protein concentration gradient, the channel was first filled with 500nM thermolysin in 5 mM Tris-HCl buffer and then the same buffer without protein was flowed into the microfluidic channel to drive the protein away from the electrode. (b) Signal generated by protein-free buffer flowed into the microchannel filled with the same 5 mM Tris-HCl buffer. The absence of detectable signal supports that the observed TIMES signal was produced by protein instead of other side effects.

[0012] FIGs 5(a)-5(f) show TIMES signal in Hepes buffer. (a)-(c). Trypsin and pABA, with K_D calculated to be 54.9 μM . (d)-(f). Thermolysin and phosphoramidon, with K_D calculated to be 27.0 nM.

[0013] FIGs 6(a)-6(f) show TIMES signal in 5mM Mops buffer. (a)-(c). Trypsin and pABA, with K_D calculated to be 30.9 μM . (d)-(f). Thermolysin and phosphoramidon, with K_D calculated to be 19.1 nM.

[0014] FIGs 7(a)-7(f) show binding between lysozyme and NAG, tested in $1\times$ PBS buffer pH = 7.4 by TIMES: (a) current vs time, (b) calculation of K_d , and (c) histogram of K_d .

Binding between lysozyme and NAG₃, tested in 1× PBS buffer pH = 7.4 by TIMES: (d) current vs time, (e) calculation of Kd , and (f) histogram of Kd .

[0015] FIGs 8(a)-8(f) show experimental current measured by TIMES with different lysozyme flow rates (30–300 $\mu\text{L}/\text{min}$) at constant lysozyme concentration of 200 μM (a);
 5 different lysozyme concentrations (200–800 μM) at constant flow rate of 300 $\mu\text{L}/\text{min}$ (b); the computation of temporal profiles of scaled flux (F) in CFD simulation as a function of dimensionless groups, Da , ε and Pe (corresponding to (c), (d) and (e) labeling on Figures, respectively). The profile (c) was computed at ($\varepsilon = 40$ and $Pe = 5000$); the profile (d) was calculated at ($\varepsilon = 40$ and $Da = 10$); the profile (e) was at $Pe = 5000$. The dimensionless time

10 (τ) was defined as $\tau = t \left(\frac{D_{\text{NAG}}}{R^2} \right)$.

[0016] FIGs 9(a)-9(e) show examples of snapshots of lysozyme before (a) and after (b) binding with NAG on Au (111) surface from atomistic simulations for 20 ns, their corresponding contours (c, d) of induced gold surface electric potential ϕ on the X - Y plane above the surface of 0.3 nm by taking average with the configurations of the last 2 ns, and the
 15 comparison of RMSD of simulated solution structures of pure lysozyme and lysozyme of the complex binding with NAG ligand (e). RMSD profiles for pure lysozyme and lysozyme of the lysozyme–NAG complex solution structures. In RMSD computation, the first conformation of the sampled trajectories was used as a reference. The area around atom #514–587 is indicated with arrows on the snapshots (b) and RMSD curves (e) respectively.
 20 The atom number is only for heavy backbone atoms. For the purpose of clarity, water and ions are excluded from the snapshots (a, b). The induced surface electric potential was computed by fixing lysozyme (or lysozyme–NAG complex) configuration to relax the system for 2 ns. The last 1 ns data including total 200 configurations was used to take the average of ϕ .

[0017] FIG 10 shows structural variance characterized by RMSD between pure lysozyme (pdb code: 1AKI) and lysozyme in lysozyme–NAG complex (pdb code: 3TXJ). The structures were taken from protein data bank. Protein of the complex was taken as a reference. The region (atom# 514–587) is indicated with arrows. The atom number is only for heavy backbond atoms.

[0018] FIG. 11 shows diagram of different boundary conditions used in the physical
 30 model.

[0019] FIG. 12 shows schematics of a microfluidic channel. Protein solution of concentration C_{A0} is injected into a microchannel of the height, h .

DETAILED DESCRIPTION

[0020] Disclosed herein, among other things, is a method, referred to as Transient Induced Molecular Electronic Spectroscopy (TIMES), to detect interactions of molecules such as protein-ligand interactions without any protein engineering or chemical modification.

5 The physics model of the TIMES signal is established and mathematical formula is generated to attain a physical insight of molecule interactions without any disturbances by molecular probes, fluorescent labels, or immobilization of molecules. For a demonstration of the functionality of this method, the TIMES signals are used to find the dissociation constants for the affinity of reactions, the shear-stress dependent adsorption time of molecules on surface, and other interesting features of interactions of molecules in native conditions. Although
10 protein-ligand binding and detection of protein-ligand complex are used in this document as examples for studying the interactions between molecules, one of ordinary skill in the art would understand that the disclosed technology, methods and devices can be applied to study the interactions between other molecules under native conditions or in a native environment.

15 [0021] Protein–ligand interaction detection without disturbances (e.g., surface immobilization, fluorescent labeling, and crystallization) presents a key question in protein chemistry and drug discovery. As a unique tool, TIMES incorporates a unique design of microfluidic platform and integrated sensing electrodes to operate in a label-free and immobilization-free manner to provide crucial information for protein-ligand interactions in relevant physiological conditions, and offers a simple and effective method to investigate
20 fundamental protein chemistry and drug discoveries.

[0022] Through experiments and theoretical simulations, it is demonstrated herein that the TIMES technique actually detects protein–ligand binding through signals generated by surface electric polarization. The accuracy and sensitivity of experiments are demonstrated
25 by precise measurements of dissociation constant of lysozyme and *N*-acetyl-D-glucosamine (NAG) ligand and its trimer, NAG₃. Computational fluid dynamics (CFD) computation is performed to demonstrate that the surface's electric polarization signal originates from the induced image charges during the transition state of surface mass transport, which is governed by the overall effects of protein concentration, hydraulic forces, and surface fouling due to protein adsorption. Hybrid atomistic molecular dynamics (MD)
30 simulations and free energy computation show that ligand binding affects lysozyme structure and stability, producing different adsorption orientation and surface polarization to give the characteristic TIMES signals. Although the protein–ligand interactions are used as examples,

the TIMES method is a general technique that can be applied to study signals from reactions between many kinds of molecules.

[0023] Functional proteins, especially those surrounded by the liquid environment, have extraordinary complexities and degrees of freedom to form 3D structures, while their biological functions are sensitive to and can be modified substantially by their binding with molecules (i.e., ligands) that are much smaller than themselves. The abilities to quantitatively and precisely characterize protein–ligand interactions are essential to understanding and controlling protein’s properties. There have been several sensing techniques for investigation of protein–ligand interactions, including surface plasmon resonance (SPR), isothermal calorimetry (ITC), biologically modified field effect transistors (BioFET), differential light scattering (DLS), fluorescence resonance energy transfer (FRET), electrophoretic mobility shift (EMSA) and small molecule microarray, etc. Most of these methods can measure binding affinity, kinetics, and other thermodynamic characteristics of protein–ligand interactions. However, there are still open and important problems not addressed by the existing methods: (i) Using fluorescent labeling on molecules in FRET, EMSA, and small molecules microarray detection methods, external modifications are added to the molecules, which could affect the binding sites or molecular structural configurations. (ii) Using surface immobilization in SPR and BioFET techniques, spatial limitation is introduced to alter the entropy of the system, which can affect the experimental results by limiting protein movements or protein folding/unfolding, and cause discrepancies from reactions in physiological conditions. (iii) Techniques such as ITC relies on heat release from the reactions have relatively low resolution, produced limited information on reaction kinetics, and face difficulties in reactions that do not generate a large amount of heat (e.g. entropy driven rather than enthalpy driven reactions). (iv) Optical methods such as DLS only work for proteins that can crystalize or produce aggregation, with other constraints on the critical temperature and concentration.

[0024] In sum, the existing methods require the formation of protein crystal or aggregate, fluorescent labeling, or surface immobilization of molecules. Given the small size of ligand molecules and the importance of protein folding in 3D space for the reactions, fluorescent labeling and molecular immobilization can introduce significant disturbances to the reactions, producing potentially incorrect or misleading results in key parameters such as reaction coefficients (e.g., dissociation constant, K_d). On the other hand, these label-free and immobilization-free methods that are currently available, such as ITC and differential

optical scattering techniques, render low throughput and limited temporal resolution, and often work only under special conditions (e.g., protein crystallization or exothermic reactions).

[0025] The method disclosed herein, Transient Induced Molecular Electronic Spectroscopy (TIMES), can detect protein-ligand binding without the above constraints. TIMES is a label-free, immobilization-free technique, and produces accurate and repeatable results with high temporal resolution. In TIMES, the readout is related to molecular interactions with the electrode surface, whereas the reaction itself is performed in the bulk space. As a method of signal readout, the TIMES signal shows the electric response of the reaction products approaching the electrode surface connected to a low-noise electric amplifier. The TIMES method measures the signal caused by the dipole moment change when protein and ligand form protein-ligand complex, breaking new grounds for studies of protein-ligand interaction. The TIMES signal has an excellent signal-to-noise ratio and timing resolution even though the difference in the molecular weight and chemical composition between protein and protein-ligand complex could be very small, sometimes less than 1%. The TIMES method produces signals related to the dipole moment and charge distribution of molecules, thus providing not only undisturbed signal in physiological conditions but also signals revealing molecular properties unattainable by and complementary with the existing methods including FRET, SPR, etc. Some characteristics and functions of the TIMES signals, include but are not limited to, measurements of reaction dissociation constants between proteins and ligands.

[0026] Disclosed herein through experiments and physical computations are four aspects: (a) The accuracy of the TIMES technique is demonstrated by measuring the dissociation constant of lysozyme protein with *N*-acetyl-D-glucosamine (NAG) and its trimer, *N,N',N''*-triacetylchitotriose (NAG₃) ligands, and showing that the dissociation coefficient of protein-ligand complex made of the same type of molecule can differ by 3 orders of magnitude. (b) Aided by an analytical model and detailed computational fluid dynamics (CFD) calculations, it is shown that the measured TIMES signal is directly proportional to the induced charge of a protein molecule (or protein-ligand complex) approaching the electrode. (c) The macroscopic level molecular transfer in a microfluidic channel is related to the microscopic molecular interfacial mass transfer by incorporating the effect of hydraulic forces and surface's biofouling (i.e., protein adsorption and desorption from the electrode surface subject to the flow induced shear stress). (d) Full-atom molecular

dynamics (MD) simulation combined with binding free energy computation is performed to elucidate the fundamentals of the electric signal, which is related to the adsorbed protein's charge distribution (such as net charge, dipole moment, etc.) and surface polarization at the microscopic level. Through these efforts, the feasibility is demonstrated and the physical foundation of the TIMES technique is established as a method to investigate protein-ligand interactions without labeling or immobilization.

TIMES Measurements of Dissociation Constant

[0027] To create a flux of protein molecules towards the electrode, a microfluidic device can be used to produce a concentration gradient along the height of the channel. In the TIMES setup (**Fig. 1a**), the microfluidic channel has two inlets, one for the buffer solution and the other for introducing the molecule of interest (i.e. protein molecule or mixtures of protein and ligand) and one outlet. In some embodiments, a single inlet may be used and the buffer solution and the molecule of interest may be introduced one after another. The entire channel is at first filled up with the buffer solution and then the molecule of interest is introduced from another inlet (**Fig. 1b**). For a laminar flow, the travel speed at the center of the channel is the greatest and approaches zero at the channel wall where the gold electrode is located (**Fig. 1c**). As a result, a concentration gradient between the center of the channel (having the highest and constant molecular concentration) and the electrode surface can be established. Such concentration gradient produced a diffusion flux for the molecules towards the electrode to produce the TIMES signal.

[0028] Along the microfluidic channel there are gold electrodes connected to an external amplifier circuit. A molecule carrying a dipole moment in the buffer solution can interact with the electric field near the solution/electrode interface within the Debye length, which is in the order of 1nm for typical ionic strength. The interfacial electric field, approximately equal to the Zeta potential divided by the Debye length according to the double-layer model can "orient" the molecule according to its charge state and dipole moment to minimize the free energy. As the molecules are oriented by the surface field, an induced dipole develops due to the mirror effect of the metallic surface. In the near field condition, dipole moment of a macromolecule such as protein has the dominant effect over the net charge of the molecule, and the alignment of the dipole moment with the surface field produces a charge transfer between the gold electrode contacting the fluid and the measurement circuit system in **Fig. 1c**. Such charge transfer induces an electric current that is amplified and converted into a voltage signal by a transimpedance amplifier (TIA). The low-noise amplifier can a transimpedance amplifier, and the transimpedance can be greater than 1 Mohm.

[0029] After converting the analog signal to digital signal through an analog-to-digital converter (ADC), the real-time signal originated by the field-induced dipole orientation of the molecules can be recorded. In the test system, protein is the only macromolecule that possesses a large dipole moment (Table 1), and all other ions in the buffer move around the protein to minimize the free energy of the system. Hence the detected signal is primarily produced by protein or protein-ligand complex near the electrode. Although in many cases the ligand molecular weight could be significantly less than the molecular weight of the protein, the formation of protein-ligand complex can alter the 3D configuration of the protein molecule, thus changing the dipole moment and charge distribution appreciably. The TIMES method monitors the native protein ligand interactions requiring no immobilization or labelling and with high temporal resolution. Next the physical principle that produces the TIMES signal is described.

Table 1 PDB-ID and calculated dipole moment of proteins

Protein	trypsin	thermolysin	<i>p</i> -ABA	phosphoramidon
Molecular Weight (D)	24.4k	34.6k	135	543
Charge (e)	6	-10	2	-2
Dipole (μ /D)	353	547	3.28	3.71

[0030] It is assumed that with sufficient ionic strength in the buffer, the “local charge neutrality” condition is satisfied when protein travels in solution without an external field (**Fig. 1d, i**). Local charge neutrality assumes that charges on the surface of protein are neutralized by the mobile counter charges in the buffer within a time scale of “dielectric relation time” equal to ϵ/σ where ϵ and σ are the permittivity and conductivity of the buffer solution. Since the dielectric relaxation time is typically 1 to 100 ns, much shorter than the time scale of interest in the measurement, the local charge neutrality condition can be satisfied everywhere in the solution (**Fig. 1d**) except for regions right next to the electrode/liquid interface where the electric field is present. When the protein molecules approach the electrode and experience the electric field from the Zeta potential, two things take place: at first those mobile ions around the protein to maintain local charge neutrality are stripped off by the E-field and find their new equilibrium distributions, and secondly the protein molecules are oriented to have their dipole moment aligned to the direction of the field (**Fig. 1d, i**). The alignment of dipole moment of protein gives rise to an induced charge flow between the electrode and the amplifier input, giving rise to a detectable signal (**Fig. 1d,**

ii). Driven by the shear stress of the laminar flow in the microfluidic channel, the protein molecules may not permanently be adsorbed to the electrode surface but detached from the gold electrode after some period of time. The departing molecules carry neighboring ions with them via electrostatic interactions and drag force, disturbing the local ion distribution and generating an overshoot in the electric signal (**Fig. 1d, iii**). Eventually, those ions carried by the departing proteins return to the electrode surface and the steady state is restored (**Fig. 1d, iv**). The TIMES signal produced by 500nM thermolysin molecules is shown in **Fig. 1e**. Similar analysis and model of protein-surface interaction have been reported before^{22, 23}. The theory and mathematical formulation of TIMES signal is described next.

[0031] It is assumed that $q(t)$ is the induced charge in the gold electrode in response to a protein molecule reaching the electrode surface. $q(t)$ can be treated as the “impulse response” or the “Green’s function” generated by a single protein (or protein/ligand complex) molecule approaching the electrode, having the unit of “Coulomb”. The net charge signal on the electrode, induced by all the protein molecules at a specific time, can be represented as

$$S(t) = A \int_0^t q(u)J(t-u)du \quad (1)$$

[0032] where A is the area of the electrode and $J(t)$ is the flux of the protein molecules at the electrode. The flux of molecule can be represented as

$$J(t) = J_+(t) - J_-(t) \quad (2)$$

[0033] where J_+ and J_- are the flux of molecular adsorption and desorption, respectively.

[0034] The expression for the protein concentration immediately next to the electrode surface, $n_i(t)$, which is related to the protein concentration outside the Debye length where the electric field is nearly zero, is calculated as follows:

$$n_i(t) = n(0, t)e^{\frac{-Ze\zeta}{kT}} = \gamma n(0, t) \quad (3)$$

[0035] where Ze is the charge of the protein and ζ is the zeta potential. Throughout the analysis, it is assumed that the protein concentration is low enough not to change the ionic strength of the buffer. Therefore, the zeta potential is not changed significantly by the protein so γ can be treated as a constant and its value is determined by the electrode material and the buffer solution.

[0036] The transport of protein across the channel thickness is governed by the equation:

$\frac{\partial n(x, t)}{\partial t} = D \frac{\partial^2 n}{\partial x^2}$ with the boundary conditions: $n(x = L, t) = n_o$ and $\frac{\partial n}{\partial x}|_{x=0} = 0$ where $x = L$ is the center of the microfluidic channel (i.e. the channel height is $2L$). The analytical solution for $n(0, t)$ at the position just outside the Debye length (i.e. E-field is nearly zero) is

obtained as

$$n(x=0, t) = \left[n_o - \sum_{M=0}^{\infty} \frac{4n_o(-1)^M}{(2M+1)\pi} e^{\frac{-(2M+1)^2 D t}{4L^2}} \right] \quad t > 0 \quad (4)$$

[0037] The detailed derivation is as follows: Assume that each time a protein hits the electrode surface, it will induce a charge on the gold electrode it contacts: $q(t)$. $q(t)$ has the unit of Coul. The protein may stay on the electrode surface or leaves the surface after a time period τ_s . The net charge signal produced by all the protein molecules at a specific time becomes

$$S(t) = A \int_0^t q(t-u)J(u)du = A \int_0^t q(u)J(t-u)du \quad (A)$$

[0038] where $J(t)$ is the net flux of protein towards the electrode, having the unit: #/Area-s.

Taking the time derivative of (1), it is obtained

$$\frac{dS(t)}{dt} = Aq(t)J(0) + A \int_0^t q(u) \left[\frac{d}{dt} J(t-u) \right] du \quad (B)$$

$\frac{dS(t)}{dt}$ is the amount of protein-induced current (unit: Amp).

[0039] The fluid is divided into two regions: region 1 covers the entire space in the microfluidic channel and region 2 is the proximity to the surface of the electrode, typically within the Debye length of the thickness of around 1 nanometer. Since there exists no electric field in region 1 and because of the laminal flow, the protein concentration away from the electrode interface is governed by the diffusion equation due to protein concentration gradient. There is

$$\frac{\partial n}{\partial t} = D \frac{\partial^2 n}{\partial x^2} \quad (C)$$

[0040] with the boundary conditions:

$$\begin{aligned} n(t, x=L) &= n_o \text{ for } t \geq 0 \\ \frac{\partial n(t, x=0)}{\partial x} &= 0 \quad \text{for } t \geq 0 \\ n(t=0, x) &= 0 \text{ for } 0 \leq x < L \end{aligned} \quad (D)$$

[0041] As shown in FIG 11, the problem in (D) is equivalent to the problem in (E) with different boundary conditions:

$$\begin{aligned}
n(t, x = L) &= n_o & \text{for } t \geq 0 \\
n(t, x = -L) &= n_o & \text{for } t \geq 0 \\
n(t = 0, x) &= 0 & \text{for } -L < x < L
\end{aligned} \tag{E}$$

[0042] Assume $u(x, t) = T(t)X(x)$ is a solution for (C)

$$\frac{1}{T(t)} \frac{dT(t)}{dt} = \frac{D}{X(x)} \frac{d^2X(x)}{dx^2} = -k$$

$$u(x, t) = e^{-kt} \left[C_k \cos\left(\sqrt{\frac{k}{D}}x\right) + E_k \sin\left(\sqrt{\frac{k}{D}}x\right) \right]$$

[0043] The general solution can be represented as

$$n(x, t) = n_o - \sum_k e^{-kt} \left[C_k \cos\left(\sqrt{\frac{k}{D}}x\right) + E_k \sin\left(\sqrt{\frac{k}{D}}x\right) \right] \tag{F}$$

5

[0044] Using the boundary conditions in (E), there is

$$\frac{\partial n(x=0, t)}{\partial x} = 0 = \sum_k \sqrt{\frac{k}{D}} e^{-kt} \left[-C_k \sin\left(\sqrt{\frac{k}{D}}x\right) + E_k \cos\left(\sqrt{\frac{k}{D}}x\right) \right]_{x=0} \quad \text{for } t > 0$$

[0045] This means $E_k' = 0$

$$n(x, t) = n_o - \sum_k e^{-kt} \left[C_k \cos\left(\sqrt{\frac{k}{D}}x\right) \right] \tag{G}$$

10 [0046] Also from (E) it is required

$$n(x = \pm L, t) = n_o - \sum_k e^{-kt} \left[C_k \cos\left(\sqrt{\frac{k}{D}}L\right) \right] = n_o \quad \text{for } t > 0$$

[0047] Then there is $\sqrt{\frac{k}{D}}L = \left(M + \frac{1}{2}\right)\pi \quad M: -\infty, \dots, \infty$

$$n(x, t) = n_o - \sum_{M=-\infty}^{\infty} e^{-\frac{(M+1/2)^2 D t}{L^2}} C_M \cos\left(\frac{(M+1/2)\pi x}{L}\right) \quad \text{for } t \geq 0$$

[0048] The above equation can be simplified as

$$n(x, t) = n_o - \sum_{M=0}^{\infty} C_M' e^{-\frac{(2M+1)^2 D t}{4L^2}} \cos\left(\frac{(2M+1)\pi x}{2L}\right) \quad \text{for } t \geq 0 \tag{H}$$

15

[0049] At $t=0$,

$$n(x, t = 0) = 0 = n_o - \sum_{M=0}^{\infty} C_M' \cos\left(\frac{(2M+1)\pi x}{2L}\right) \quad \text{for } -L < x < L \tag{I}$$

$$C_M' = \frac{n_o \int_{-L}^L \cos\left(\frac{(2M+1)\pi x}{2L}\right) dx}{\int_{-L}^L \cos^2\left(\frac{(2M+1)\pi x}{2L}\right) dx} = \frac{4n_o(-1)^M}{(2M+1)\pi} \tag{J}$$

[0050] Hence

$$n(x, t) = n_o - \sum_{M=0}^{\infty} \frac{4n_o(-1)^M}{(2M+1)\pi} e^{-\frac{(2M+1)^2 D t}{4L^2}} \cos\left(\frac{(2M+1)\pi x}{2L}\right) \quad (K)$$

[0051] To find out $n(x=0, t)$

$$n(0, t) = n_o - \sum_{M=0}^{\infty} \frac{4n_o(-1)^M}{(2M+1)\pi} e^{-\frac{(2M+1)^2 D t}{4L^2}} \quad (L)$$

5 [0052] Note that (L) is the protein concentration in region 1 where there is no electric field. At the surface of the electrode, the protein concentration can be approximated as

$$n_i(t) = n(0, t) e^{-\frac{Ze\zeta}{kT}} = \left[n_o - \sum_{M=0}^{\infty} \frac{4n_o(-1)^M}{(2M+1)\pi} e^{-\frac{(2M+1)^2 D t}{4L^2}} \right] e^{-\frac{Ze\zeta}{kT}} = \gamma n(0, t) \quad (M)$$

$$\gamma = e^{-\frac{Ze\zeta}{kT}}$$

[0053] where Ze is the charge of the protein and ζ is the zeta potential. It is assumed that
10 the protein concentration is low enough not to change the ionic strength of the buffer. Therefore the zeta potential is not changed significantly by the protein.

[0054] Note that $n(x=0, t) = \left[n_o - \sum_{M=0}^{\infty} \frac{4n_o(-1)^M}{(2M+1)\pi} e^{-\frac{(2M+1)^2 D t}{4L^2}} \right] \sim n_o$ if the time of interest is greater than $\frac{4L^2}{D}$ where D is the diffusivity of protein (or protein-ligand complex).

[0055] In equation (2),

$$15 \quad J_+(t) = K_+ n_i(t) (1 - \theta) \quad (5)$$

K_+ has the unit of velocity.

θ : fraction of monolayer deposition $0 \leq \theta \leq 1$.

$$J_-(t) = K_- \theta \quad (6)$$

K_- has the unit of flux (1/s-area).

20 [0056] At equilibrium (i.e. $t \rightarrow \infty$),

$$J_+(\infty) = K_+ n_i(\infty) (1 - \theta_o) = K_+ n_o \gamma (1 - \theta_o) = K_- \theta_o \quad (7)$$

[0057] Using the relations in Eqs. 5-7,

$$\theta(t) = \frac{\int_0^t J(u) du}{\int_0^\infty J(u) du} = \frac{K_+ \int_0^t [n_i(u)(1-\theta)\theta_o - n_i(\infty)(1-\theta_o)\theta] du}{\int_0^\infty J(u) du} \quad (8)$$

[0058] Under the approximation that the time scale of interest is significantly longer than
25 the diffusion time (i.e. $t > \frac{4L^2}{D}$), Eq. (8) can be approximated as

$$\theta(t) \sim \frac{K_+}{\theta_o} n_i(\infty) \frac{\int_0^t [\theta_o - \theta] du}{\int_0^\infty J(u) du} = \left(\frac{K_+ \gamma n_o}{\int_0^\infty J(u) du} \right) \int_0^t \left[1 - \frac{\theta}{\theta_o} \right] du \quad (9)$$

[0059] And the induced charge on the electrode by a protein molecule is obtained as

$$q(t) = \left[\frac{1}{A(K_+ \gamma n_o)} \right] \left[i(t) + \frac{1}{\tau_s} \int_0^t i(u) du \right] \quad (10)$$

$$\tau_s = \frac{\theta_o \int_0^\infty J(u) du}{K_+ \gamma n_o} = \frac{\int_0^\infty J(u) du}{K_- + K_+ \gamma n_o} \quad (11)$$

[0060] where n_o is the protein concentration at equilibrium, and τ_s has the physical meaning of the average dwelling time or surface adsorption time for a protein (or protein-ligand complex) molecule on the surface of electrode.

[0061] From (10), it is shown that the transient induced molecular current, $i(t)$, can be used to find (a) the induced charge, $q(t)$, by each molecule when approaching the electrode surface and (b) the surface adsorption time (τ_s) of the molecule on the electrode surface without any molecular labeling or surface immobilization.

[0062] The concepts using a protein-ligand pair are demonstrated as an example. Thermolysin is a 34.6 KD thermostable metalloproteinase produced by the Gram-positive bacteria *Bacillus thermoproteolyticus*. It preferentially cleaves at the N-terminus of the peptide bonds containing hydrophobic residues such as leucine, isoleucine, and phenylalanine. Phosphoramidon was isolated from *Streptomyces tanashiensis*, which inhibits thermolysin specifically. The normalized induced charge, $q(t)$, by thermolysin before and after binding with phosphoramidon under different flow rate is shown in **Figs. 2a** and **2b**. The shear stress dependent surface adsorption time of thermolysin before and after binding with phosphoramidon is compared in **Fig. 2c**.

[0063] From Fig. 2a, the surface adsorption time of thermolysin decreases monotonically with increasing flow rate because a higher flow rate in the microfluidic channel produces greater shear stress according to Poiseuille's Law to remove protein from the electrode surface, thus reducing the average adsorption time of protein molecules on the electrode. The same trend was observed for thermolysin/ phosphoramidon complex (Fig. 2b). However, thermolysin and thermolysin/phosphoramidon show different adsorption time dependence on the flow induced shear stress, as shown in Fig. 2c. The results provide clear evidences that in spite of very similar molecular weight and size of the protein and protein/ligand complex, the binding strength of the two molecules to the electrode show appreciable differences, manifested in the adsorption times measured from the TIMES signals.

[0064] Under the condition that the system consists of more than one type of molecules (e.g. coexistence of protein, ligand, and protein-ligand complex), then the measured TIMES signal can be further approximated by (12) when the second term in (10) becomes negligible. This approximation is valid when the time of concern is shorter than the protein adsorption

time τ_s .

$$i(t) \sim A \sum_i n_{o,i} v_{s,i} \gamma_i q_i(t - t_{oi}) \quad \text{where } t_{oi} = \frac{4L^2}{D_i} \quad (12)$$

[0065] In the case of first-order reaction: *Ligand + Protein* $\leftrightarrow$ *PLcomplex*

$$K_D = \frac{n_L n_P}{n_C}$$

[0066] where n_L, n_P, n_C represent the equilibrium concentration of ligand, protein, and protein-ligand complex, respectively.

[0067] Assuming all t_{oi} 's are short compared to the timing resolution of the measurement system, (12) can be approximated as

$$i(t) \sim A [n_{o,p} v_{s,p} \gamma_p q_p(t) + n_{o,L} v_{s,L} \gamma_L q_L(t) + n_{o,c} v_{s,c} \gamma_c q_c(t)] \quad (13)$$

[0068] Before reaction, the initial protein and ligand concentrations are assumed to be x and y, respectively. After the equilibrium is reached,

$$n_{o,p} = x - z, \quad n_{o,L} = y - z, \quad n_{o,c} = z$$

[0069] Substituting these relations into (13),

$$i(t) \sim A \left[[(x - y - K_D) + \sqrt{(x + y + K_D)^2 - 4xy}] G_p(t) + \right. \\ \left. [(y - x - K_D) + \sqrt{(x + y + K_D)^2 - 4xy}] G_L(t) + \right. \\ \left. [(x + y + K_D) - \sqrt{(x + y + K_D)^2 - 4xy}] G_c(t) \right] \quad (14)$$

[0070] In (14) the TIMES signal $i(t)$ is measured under given values of x and y are known from different mixtures of protein and ligand. Since there are four unknowns: K_D , $G_p(t)$, $G_L(t)$, and $G_c(t)$ in (14), TIMES signals in 4 different combinations of protein ligand concentrations (e.g. protein only, ligand only, 1:2 and 2:1 protein/ligand ratios) need to be measured to solve these unknowns. Among the four unknowns, only K_D is time independent, hence ideally the same value of K_D at each time point should be obtained when the unknowns are solved. However, when noise added to (14) as a random variable, it causes fluctuation of K_D obtained at each time point. At a sampling rate of 1ms over 1s period, 1000 values of K_D can be obtained. Therefore a histogram of K_D value can be generated to help determine its mean value to minimize the effect of noise. It is recommended that at least in two of 4 experimental conditions, x and y should be in the same order of K_D (i.e., $0.1 K_D < x, y < 10 K_D$) to minimize the influence of noise. When there is no prior knowledge about the order of magnitude of K_D , x and y can be chosen arbitrarily and the choice can be examined from the histogram of K_D . Both the obtained value of K_D and the distribution of the histogram can clearly indicate the appropriateness of the initial choice. Normally in one iteration, the proper

range of x , y (i.e., protein and ligand concentration before reaction) that yields accurate K_D can be found.

[0071] TIMES signals are used to find the dissociation constant of protein-ligand interactions nonmetal-dependent and metal-dependent enzymes: (a) trypsin and *p*-aminobenzamidine (*p*-ABA), (b) thermolysin and phosphoramidon.

[0072] Trypsin is a serine protease that hydrolyzes proteins in the vertebrate digestive system. TIMES signals were measured by adding different amounts of *p*-ABA to the trypsin solution (**Fig. 3a**). What particularly intriguing is that samples with different amounts of *p*-ABA generate TIMES signals that are markedly different from the trypsin signal, indicating that formation of trypsin/*p*-ABA complex can appreciably alter its charge distribution and dipole moment even though *p*-ABA is a much smaller molecule than trypsin. By measuring the TIMES signals produced by different ratios of trypsin and *p*-ABA in the mixtures, Eq. (3) can be used to (a) find the impulse response or Green's function $q(t)$ for the protein-ligand complex as well as the reaction dissociation coefficient K_D at each time point, as shown in

Fig. 3b. The spikes in the plot of K_D vs. time are caused by noise added to Eq. (14). Those spikes are outliers in the histogram plot of K_D in **Fig. 3c**, and the most likely value in the histogram is used to be the value of K_D , which is found to be 34.7 μ M. Three repeated runs were performed, and the averaged value agrees with the reported K_D from literatures (**Table 2**). The minor difference could be due to different buffers and pH value in different experiments (5mM Tris-HCl buffer at pH=7.4 was used). In the experiment with another protein ligand pair: thermolysin and phosphoramidon, the TIMES signals were obtained as shown in **Fig. 3d**, with K_D calculated in **Figs. 3e, 3f** to be 33.1 nM. Repeated experiments showed the averaged K_D value to be 32.1 nM, which is in excellent agreement with the reported values (**Table 2**). The tests were also performed in Hepes buffer and Mops buffer under same temperature and pH, with results shown in **Figures 4-6**. The above examples demonstrate that from the TIMES signals, one can investigate protein-ligand binding with a wide range of equilibrium dissociation constants from μ Ms to nMs. Limited by the noise of transimpedance amplifier, the lowest dissociation constant can be measured with the current setup is ~ 1 nM.

Table 2 Summary of measured dissociation constant from TIMES and literatures

Protein-ligand pairs	Measured K_D from TIMES	Reported K_D from literatures
Trypsin and <i>p</i> -ABA	39.1 $\pm$ 3.6 μ M	12 μ M (Markwardt et al.) ²⁴ 19 μ M (Malanikova et al.) ²⁵

Thermolysin and phosphoramidon	32.1±1.9 nM	23 nM (Kitagishi et al. 1984) ²¹ 28 nM (Komiyama et al. 1975) ²⁶
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Table 3 Summary of measured dissociation constant of two protein ligand pairs from TIMES in three different buffers

Protein-ligand pairs	Buffers	Measured K_D from TIMES
Trypsin and <i>p</i> -ABA	Tris	39.1±3.6 μ M
	Hepes	54.9±7.1 μ M
	Mops	30.9±4.0 μ M
Thermolysin and phosphoramidon	Tris	32.1±1.9 nM
	Hepes	27.0±2.6 nM
	Mops	19.1±1.8 nM

- 5 [0073] TIMES experimental measurements of protein-ligand binding dissociation constant (K_d) are shown in another example. Figure 7 shows the measured results of the dissociation constants between lysosome and two ligands: NAG and NAG₃. According to the analytical model disclosed herein, the measured signal of TIMES system can be represented as

$$10 \quad I(t) \sim A \sum_i n_{o,i} K_{+,i} \gamma_i q_i(t - t_{oi}) = A \sum_i n_{o,i} K_{+,i} Q_i(t - t_{oi}) \quad (15)$$

- with the electrode area A , volume concentration of molecules (protein, ligand, or protein-ligand complex) $n_{o,i}$ rate of molecular adsorption to the electrode surface $K_{+,i}$, the coefficient $\gamma_i = \exp\left(\frac{-Z_i \phi_s^*}{eV}\right)$ with ζ being the zeta potential, molecular induced charge as a function of time $q_i(t-t_{oi})$, $Q_i(t-t_{oi}) = \gamma_i q_i(t-t_{oi})$, and diffusion time $t_{oi} = \frac{4L^2}{D_i}$ for the molecule to transport transversely toward the electrode.

[0074] Utilizing electric signals for ligand, protein, and protein-ligand complex, protein-ligand dissociation constant can be obtained to estimate protein-ligand dissociation coefficient (K_d),

$$K_d = \frac{n_L n_P}{n_C} \quad (16)$$

- 20 [0075] where n_L , n_P , and n_C represent the bulk concentration of ligand, protein, and complex, respectively, in the unit of mol/L. Figure 7a shows TIMES signals for different

ratios of lysozyme and NAG. A total of 4 unknowns are to be found: the time- dependent induced charge response by the protein, ligand, and protein–ligand complex, namely, $Q_P(t)$, $Q_L(t)$, and $Q_{P-L}(t)$ in (15), and the dissociation constant, K_d . To find all these values, an experiment was performed by flowing four samples through the device: for example, samples containing protein only, ligand only, and 1:2 and 2:1 protein to ligand concentration ratios before reaction. From the measured TIMES signals of the 4 samples and using (15) and (16) one can find unique solutions for $Q_P(t)$, $Q_L(t)$, $Q_{P-L}(t)$, and K_d at each time point. Since out of the 4 unknowns only K_d is time independent, a histogram for K_d found at each time point can be obtained. This histogram can produce not only the value of K_d but also the quality of the measurement, since a reliable measurement should yield a tight distribution of the K_d value. In other words, in one single set of measurements, K_d can be measured 1000 times over a duration of 1 s at a sampling rate of 1000 s⁻¹. Figure 7b shows the dissociation constant, K_d , and Figure 7c shows the histogram of K_d obtained from the method described above. It was found that the most likely value of dissociation constant for lysozyme and NAG is 12.59 mM. The experiment was repeated three times, and the averaged dissociation constant estimated from TIMES is summarized in Table 4, which also includes previously published value(s) for comparison. The large dissociation constant (i.e., on the order of 10 mM) suggests that the binding between lysozyme and NAG is very weak. The binding can be strengthened significantly by using the trimer of NAG (NAG₃), as shown in Figs 7(d)-7(f). The histogram shows that the value of the dissociation constant between lysozyme and NAG₃ is 39.81 μM (Fig 7(f)), which is nearly 3 orders of magnitude lower than the value between lysozyme and NAG. The measured value is found to be close to the reported value by other detection methods (see Table 4). It is noted that, besides the complex of lysozyme–NAG or NAG₃, the TIMES techniques can be applied to measure the binding constant of different complexes, e.g., trypsin and *p*-aminobenzamidine, and thermolysin and phosphoramidon, and very precise values can be obtained.

Table 4 Comparison of Lysozyme–Ligand Dissociation Constant, K_d , between the TIMES Method and the Previously Published Results Achieved by Other Methods

ligand	K_d			
	TIMES	lit. reps		
NAG	12.59 mM	16 mM ⁴⁰	24 mM ⁴⁵	7 mM ⁴⁶
NAG ₃	39.81 μM	39 μM ⁴⁴	38.3 μM ⁴⁷	19.6 μM ⁴⁴

[0076] The TIMES signal waveform shown in Figures 7a and 7d demonstrates that the signals produced by protein and protein–ligand appear to be obviously different even though the size, molecular weight, and dipole moment of ligand molecule are orders of magnitude smaller than those of protein. A possible explanation for why a protein bound with a small
5 ligand can produce significant change in the TIMES signal is that ligand binding can alter the folding and/or orientation of protein. Atomistic simulations are disclosed below.

CFD Computations

[0077] The TIMES electric signal arises from molecular interactions with the electrode surface and is also affected by external hydraulic forces and surface fouling due to protein
10 adsorption. To further investigate the mechanism, both experiments and CFD computations are used. Figure 8a presents the measured induced currents under different flow rates. By changing the flow rate of lysozyme in the microfluidic channel, the shear stress is changed and so is the driving force to pull the protein away from the electrode. Therefore, the average
15 dwelling time for a protein molecule on the electrode surface is reduced with increased flow rate, which is consistent with the data in Figure 8a. Different lysozyme concentrations are also examined as shown in Figure 8b. By flowing different protein concentrations from 200 μM to 800 μM into the microfluidic channel, the signal intensity increases linearly with the
20 concentration as shown in (15) and then saturates as the concentration becomes very high. It is believed that the signal intensity saturation is caused by Coulomb repelling and steric hindrance of molecules near the electrode surface. In other words, the surface adsorption rate K_+ in (15) is no longer constant, but decreases with increasing molecular concentration. Finally, all the temporal profiles of the TIMES signal (see Figures 8a, and 8b) exhibit a
25 similar characteristic waveform: starting with a fast increase in the signal intensity and followed by a decrease that often displays a negative overshoot before returning to the zero value. Such a general pattern of the waveforms suggests that the waveform follows the protein flux toward the electrode surface. Each time a protein reaches and leaves the electrode, the TIMES signal is produced.

[0078] Fluidic dynamic simulation of the mass transfer process inside a microfluidic channel was investigated. CFD computation can be performed with a fluid dynamics model,
30 which consists of diffusion, fully developed laminar flow convection, and surface reactions. Protein–surface interactions are generally significantly stronger than protein–protein interactions, e.g., the lysozyme–Au(111) surface binding free energy (~ 59 kT) measured from the potential of mean field profile by the umbrella sampling method,⁴⁸ compared to

lysozyme-lysozyme interaction energy (~ 0.93 kT) incorporating the hydration and ion effects implicitly through Debye-Hückel theory. Due to the strong surface-protein interactions, it is conceived that the substrate gold surface is covered with a layer of tightly adsorbed proteins and then floppy multilayer adsorption is built up. To simplify the analysis, a Langmuir adsorption model is adopted, in which the effect of surface jamming limit packing is incorporated and only the first-layer adsorption is considered (See (23) in Example 3). Most of the previous studies⁵⁰⁻⁵² focused on the steady-state adsorption behavior inside a microfluidic channel involve surface adsorption or reactions; whereas the transition state is described herein to interpret the result from TIMES experiments. The equations as well as the initial and boundary conditions are scaled in order to reveal the dimensionless parameters governing the system and to explain the general mechanism. A detailed description of the simulation model and its scaled form are provided in the working examples.

[0079] To analyze the effect of the surface reactions, convection, and diffusion on the scaled surface flux (F) in the transition state, dimensionless groups, Damköhler (Da), relative concentration between the bulk and the fully saturated surface (ϵ), and Péclet (Pe) are introduced,

$$F = \frac{dC_A}{dt} \left(\frac{h^2}{D_{AB} C_{A0}} \right) \quad (17)$$

$$Da = K_{ads} C_{A(max)}^* h / D_{AB} \quad (18)$$

$$\epsilon = \frac{C_{A0} h}{C_{A(max)}^*} \quad (19)$$

$$Pe = Uh / D_{AB} \quad (20)$$

[0080] with the initial concentration C_{A0} of protein solution before entering the microchannel, the bulk protein concentration C_A inside the microchannel, protein diffusion coefficient D_{AB} , microchannel height h , adsorption rate K_{ads} , maximum surface adsorption amount $C_{A(max)}^*$, and the average bulk velocity U . The reduced number Da represents the ratio of the surface reaction rate and the diffusion rate, and Pe stands for mass transfer rate ratio of convection and molecular diffusion.

[0081] Figure 8c shows that when there is no protein adsorption (i.e., $K_{ads} = 0$ or $Da = 0$), no negative overshooting occurs. As Da increases and the value of $Pe (= 5000)$ is fixed, both the intensities of flux peak and negative overshooting increase. The results suggest that

the surface adsorption can enhance the mass flux toward the surface. When the surface adsorption amount goes beyond the threshold amount, the extra accumulated amount joins the bulk phase, which leads to the signal's negative overshooting. Figure 8e presents the concentration effects on the scaled surface flux (F) temporal profiles at different Da . The literature shows that Da can vary from the order of ~ 0.1 to ~ 10 . If the surface fouling reaction is strong (e.g., $Da = 20$), at a constant Pe , the surface flux can be scaled by the concentration. However, for the weak surface fouling reactions (e.g., $Da = 0.1$), the surface flux is not proportional to the concentration increase and the flux reaches its maximum value and declines since the mass transport is limited by surface reactions. Figure 8d shows the effect of Pe on the scaled flux at a constant ε and Da . Here Pe varies in the range of 10^2 – 10^4 , which is the order of magnitude suggested by previous study. One can observe that when Pe increases (i.e., higher flow speed), the period of transition state shortens, whereas flux intensity and overshooting phenomena become magnified. It indicates that strong convection can enhance mass transfer flux to the surface, due to the large concentration gradient normal to the surface. The results disclosed herein demonstrate that the reduced-unit flux (Figures 8d, 8e) from CFD is qualitatively consistent with the experimental results of electric current (Figures 8a, 8b). The similarity of their trends illustrates that TIMES electric signal is introduced in the transition state. The CFD simulation may offer good explanations for the negative overshooting of the TIMES signal by attributing this phenomenon to protein adsorption kinetics. The overshooting on protein adsorption kinetics can also be visible in the previous experimental measurements of lysozyme adsorption on the C16 hydrophobic self-assembled surface (SAMs) by using total internal reflectance fluorescence (TIRF).

Atomistic Simulations

[0082] To interpret the surface's electric response at the molecular level, hybrid atomistic simulations are employed. MD simulations are first carried out to simulate the solvation structures of lysozyme and lysozyme–NAG complex. Next, with the solvation structures, the adsorbed lysozyme and lysozyme–NAG complex are predicted by using hybrid MM/PBSA and full-atom MD simulation according to a previously established protocol. Previous studies show that it is computationally expensive to perform full-atom MD simulations to predict protein adsorption in explicit water, and the simulation results are highly dependent on the protein initial orientation, due to the large molecular size and slow rotational motion. Therefore, MM/PBSA can be performed to predict the initial orientation of lysozyme on Au(111) surface at a fixed protein–surface distance by treating protein as

a rigid body in an implicit water environment. Then full-atom MD simulations are carried out to relax protein conformation on polarizable Au(111) surfaces.

[0083] MM/PBSA computations show that, before binding with ligand NAG, the lysozyme molecule most likely “lies down” with residues (Ile78–Asn93) contacting the gold surface, whereas the lysozyme–NAG complex most possibly “stands up” with residues (Asn65–Asn74) close to the Au(111) substrate surface. After 25 ns further relaxation with MD simulations, both protein and protein–ligand complex undergo slight conformational changes. Figures 9a and 9b compare the final different conformations of pure protein and the complex. Consequently, their dipole moment directions are very different leading to distinct gold surface polarization. For a pure lysozyme, the angle between the dipole moment vector and positive Z-axis normal to the surface is $(90.36 \pm 4.98)^\circ$, while for NAG–lysozyme, the angle is $(27.86 \pm 8.10)^\circ$. To characterize the gold surface polarization, the X – Y plane above the gold substrate above the top of the gold surface ($8.075 \times 7.992 \text{ nm}^2$) is divided into 17×17 grids. At each grid point of the position $\vec{r}$, the electric potential ϕ ($\text{kJ mol}^{-1} \text{e}^{-1}$) of the gold surface’s image charges is calculated by summing up the Coulombic interactions over all surface atoms N ,

$$\phi = f \sum_{i=1}^N \frac{q_i}{|\vec{r} - \vec{r}_i|} \quad (21)$$

[0084] with electric conversion factor $f = 138.935 \text{ kJ mol}^{-1} \text{ nm e}^{-2}$ and the surface atom i position $\vec{r}_i$. The electric potential contour shows the overall effect of protein’s charge distribution as well as all other contributions from solvent environment, i.e., hydration water and ions. As shown in Figure 9c, 9d, different contours of induced electric potential resulting from gold substrate surface image charges are detected for the adsorbed pure lysozyme and lysozyme–NAG complex. To further investigate the reason for different lysozyme adsorption orientations before and after ligand-binding, root-mean-square displacement (RMSD) of protein’s heavy backbone excluding hydrogen atoms (see Figure 9e) is introduced to quantify protein structural stability. By using MD simulation trajectories of both pure lysozyme and lysozyme–NAG complex, RMSD is computed after an optimal overlap, where each instantaneous structure is translated and rotated to superimpose the reference structure. From RMSD profile of pure lysozyme and NAG–lysozyme complex, it is shown that compared to the lysozyme solvation structure, lysozyme bound with the NAG ligand displays smaller structural fluctuations, particularly around the area (atom

#514–587) corresponding to the adsorption region (Asn65–Asn74) for the complex. For further verification, experimental structure data from the Protein Data Bank is examined. RMSD is computed to compare structures of both pure protein and protein of a complex. The same large variance is also detected at that particular area, i.e., Asn65–Asn74 (see Figure 10).

5 Previous studies showed that a gold surface has a large surface energy. Protein's slight structural rearrangement can affect protein adsorption orientation due to the strong dehydration free energy of the gold surface. For a lysozyme molecule, sulfur atoms are not exposed to the solvent environment. Therefore, the possibility of thio (Au–S) interaction between protein and gold surface can be excluded, particularly at the earlier stage when a
10 protein molecule reaches the gold surface. Given the aforementioned analysis, it can be concluded that the binding of NAG results in the variance in lysozyme structural stability and hence affects protein dipole moment direction, which induces different electric responses as the detected TIMES signal.

[0085] As disclosed herein, the technique of Transient Induced Molecular Electronic
15 Spectroscopy (TIMES) can detect protein-ligand binding in aqueous phase without fluorescent labeling or surface immobilization of molecules. The TIMES technique allows the study of undisturbed interactions between protein and ligand. The method is based on experiment and physical computations, and is established partly on the principle that protein-ligand binding can result in detectable changes in protein's charge distribution and dipole
20 moment even though the protein-ligand complex has nearly the same molecular weight and chemical makeup as the protein itself. The physics of the signals are mathematically formulated to make the TIMES technique not only a qualitative tool but also a quantitative method to analyze the protein interactions. TIMES method can produce, for example, measurements of dissociation constant for protein-ligand binding and shear-stress dependent
25 adsorption time for protein and protein/ligand complex. The TIMES method can also be used to study protein folding, binding kinetics, protein-protein interaction, protein-aptamer interaction, and other properties important for drug discovery and protein chemistry.

[0086] As described herein and demonstrated in the working examples, the TIMES
method can accurately measure the dissociation constant for lysosome-NAG and lysozyme-
30 NAG₃ interactions and demonstrated that, compared to monomer NAG, trimer NAG₃ can enhance the binding with lysozyme by nearly 1000 times. The theory presented herein was further examined experimentally with different concentrations and flow rates of protein in a microfluidic channel as well as theoretically by performing CFD and atomistic simulations.

[0087] The CFD simulations suggest that attractive protein–electrode force and repulsive shear force in a microfluidic channel determine the surface flux of protein, which gives the general waveform of the TIMES signal characterized by a positive peak followed by a negative overshoot before returning to the baseline. With the induced electrical signal from proteins dwelling on the electrode surface, the effects of protein and ligand concentration and hydraulic shear stress on protein–ligand binding and proteins’ kinetic transport at the water–surface interface inside a microfluidic channel were investigated. The efficient hybrid MM/PBSA calculations and MD simulations predict the most probable adsorption orientations of protein and protein–ligand complex and the subsequent surface polarization. The results indicate that protein configuration change due to ligand binding contributes to the TIMES signal and enables the method to detect protein–ligand interactions and find the reaction dissociation constant. Although protein–ligand interactions are used as examples, the technique and general principle of TIMES can be easily extended to study interactions of different kinds of molecules as an effective tool to characterize biomolecular reactions in conditions closest to their native environments. Such examples include interactions between proteins and nucleic acids, proteins and aptamers, aptamers and small (drug) molecules, etc. Additionally, disclosed herein is the TIMES method used for characterization of protein-ligand interactions with a single binding site. More complicated systems involving multiple-ligand binding can be developed and used based on this disclosure.

[0088] In some embodiments, the detection of protein-ligand binding may thus be used laboratory testing, drug discover, understanding and calibrating reactions and other research and development purposes. Using the amount of protein-ligand binding and by calculating rate at which the binding occurs, e.g., drug delivery and dosage calculations may be performed.

WORKING EXAMPLES

[0089] The following examples illustrate various embodiments of the technology disclosed in this document. By no means the following examples limit the scope of the invention in any way.

Example 1 TIMES Experiment Setup

[0090] Device fabrication: The TIMES system consists of a microfluidic channel to allow

the biomaterials to flow through, a pair of gold electrodes on the floor of the microfluidic channel as the sensing electrode and ground electrode, two inlets to inject molecules of interest and buffer respectively, and a transimpedance amplifier with its input connected to the gold sensing electrode. The device was fabricated on a 1mm thick glass slide (VWR).

5 Before fabrication, the glass slide was cleaned in acetone, methanol, and isopropyl alcohol (IPA) for 5 minutes in each chemical sequentially with sonication, and blow-dried by nitrogen gas. The glass slide was first lithographically patterned by NR9-1500PY photoresist (Futurrex, USA). After deposition of 100 nm titanium (Ti) and 200 nm gold (Au) films on the glass slide using a sputtering system (Denton Discovery 18, Denton Vacuum, LLC), lift-off

10 process with low power sonication was employed to remove the photoresist to form the Ti/Au patterns. Each Ti/Au sensing area was $1 \times 1 \text{ mm}^2$ with an extended area outside of the channel to allow connection to the external circuits via soldered wires. The microfluidic channel was fabricated using soft lithography process. The mold was fabricated on a 4 inches Si wafer. After the standard wafer cleaning process, a layer of 30 μm thick SU8-2050 (Microchem)

15 photoresist was spun on the silicon wafer and patterned photolithographically to form the SU8 mold. Uncured polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, MI) was poured onto the SU8 mold and cured in a 65 °C oven. Two parts of PDMS (base: curing agent=10:1) were well mixed and degased in a vacuum chamber, and were poured onto the SU-8 mold and cured at 65 °C for 4 hours. After curing, PDMS was separated from the SU-8

20 mold and holes were punched on the PDMS to form inlets and outlets. Finally, after UV ozone treatment the PDMS part and the Ti/Au patterned glass slide were aligned under microscope, and baked on 120°C hotplate for half an hour to ensure bonding, which produced the device in **Fig. 1**.

[0091] Experiment setup: The two inlets of microfluidic channel were connected to two

25 syringes (BD plastic) that contained buffer and buffer with dissolved protein/ligand. The flow rates of the syringes were controlled by programmable syringe pumps (Pump 11 elite, Harvard Apparatus). One gold sensing area within the microfluidic channel was connected electrically to the input of a low noise transimpedance amplifier (SR570, Stanford Research System, Inc.), and the other gold pad within the microfluidic channel was connected to the

30 ground of the circuit. The transimpedance of the amplifier was set at 100 M Ω , and the voltage output of the amplifier was digitized by a DAQ board (National Instrument USB-6251). The data were recorded and filtered using LabVIEW Signal Express, at a sampling rate of 1 kHz. The primary sources of noise are interference from the environment and thermal noise of transimpedance amplifier. Using an electro-magnetic shielded chamber, an improved

amplifier with lower thermal noise, and digital filters (Labview), the signal-to-noise ratio can be improved to allow the measurement of reactions with very low (e.g., pM) dissociation constant.

[0092] All the components in the setup, including the stainless steel needles connecting the syringes and the inlets of the microfluidic channels were grounded electronically. Before the experiment, the microfluidic channel was flushed and filled with buffer injected from both inlets (Fig. 1b, i). Then one of the inlets was replaced with protein (or other molecules of interest) laden buffer to fill up the channel, as shown in Fig. 1b, ii. Then the syringe pump that drove the protein was stopped and the other syringe pump driving the buffer was turned on to wash the channel. The above procedure completed the conditioning of the system before test, leaving a liquid interface between protein and buffer solution near the input of the channel, as shown in Fig. 1b, iii. To start the experiment, the flow of the buffer was stopped and the protein laden solution flowed through the microfluidic channel (Fig. 1b, iv). The TIMES signal was generated as shown in Figs. 1(c)-1(e).

[0093] Data analysis: After testing protein, ligand, and different ratios of protein and ligand mixture, signals were processed by applying the physical model discussed below to attain such information as induced charge response $q(t)$, protein (or protein/ligand complex) adsorption time τ_s , and dissociation constant K_D . The physical model was implemented in Matlab.

[0094] Unless mentioned otherwise, 5 mM Tris-HCl buffer was used for all the experiments reported in this disclosure, including the control experiments described in the following. To show that protein flux from a concentration gradient contributes to the TIMES signal, a reverse concentration gradient for trypsin was created by first filling the channel with trypsin and then flowing buffer into the channel to drive the trypsin away from the electrode. By reversing the protein concentration gradient, the polarity of the TIMES signal was also reversed (Fig. 4a).

[0095] Negative control experiment was conducted by introducing Tris-HCl buffer into a Tris-HCl buffer filled channel. The absence of any detectable signal (Fig. 4b) suggests that no signal was produced by artifact such as temperature gradient or flow-induced shear stress.

[0096] A more complete list of dipole moments of protein can be found in Protein Dipole Moments Server (<http://dipole.weizmann.ac.il/>) and Pitt Quantum Repository Molecular Database (<https://pqr.pitt.edu/>).

Example 2 Biomolecule Test on TIMES

[0097] Before the test, the microfluidic channel was first filled with buffer from inlet. Chosen amounts of protein and ligand were dissolved in buffer solution before the test. For samples that contained both protein and ligand molecules, the samples were set aside for 3 h before the test to ensure that the reaction had reached the equilibrium state. All the measurements were conducted at room temperature, and each test was repeated three times to confirm repeatability. After each test, the device was washed with buffer to remove any molecule residues in the microfluidic channel or on the electrode surface. The protein and ligand binding experiments were performed in $1\times$ PBS buffer at pH = 7.4. The data obtained after the amplifier and ADC were low pass filtered digitally in Matlab to remove noise, and the dissociation constant K_d was calculated and plotted with Matlab. TIMES dissociation constant (K_d) estimation is detailed below. The protein experiment under different flow rates and concentrations was performed in 50 mM Tris-HCl buffer at pH = 7.4.

[0098] The measured signal of TIMES system can be represented as

$$i(t) \sim A \sum_i n_{o,i} K_{+i} \gamma_i q_i(t - t_{del}) \quad \text{where } t_{del} = \frac{4L^2}{D_i} \quad (i)$$

[0099] In the case of first-order reaction:

Ligand + Protein $\leftrightarrow$ *PLcomplex*

$$K_d = \frac{n_L n_P}{n_C} \quad (ii)$$

$$i(t) \sim A [n_{o,P} K_{+P} \gamma_P q_P(t) + n_{o,L} K_{+L} \gamma_L q_L(t) + n_{o,C} K_{+C} \gamma_C q_C(t)] \quad (iii)$$

[00100] Define: $G_P(t) = K_{+P} \gamma_P q_P(t)$ $G_L(t) = K_{+L} \gamma_L q_L(t)$ $G_C(t) = K_{+C} \gamma_C q_C(t)$

[00101] Before reaction, the initial protein and ligand concentration are assumed to be x and y , respectively. After the equilibrium is reached,

$$n_{o,P} = x - z, \quad n_{o,L} = y - z, \quad n_{o,C} = z$$

$$K_d = \frac{(x-z)(y-z)}{z} \quad (iv)$$

[00102] Solving z from (iv),

$$n_{o,C} = z = \frac{(x+y+K_d) - \sqrt{(x+y+K_d)^2 - 4xy}}{2} \quad (v)$$

$$n_{op} = x - z = \frac{(x-y-K_d) + \sqrt{(x+y+K_d)^2 - 4xy}}{2} \quad (v2)$$

$$n_{ol} = y - z = \frac{(y-x-K_d) + \sqrt{(x+y+K_d)^2 - 4xy}}{2} \quad (v3)$$

[00103] Substituting (v) into (iii)

$$\begin{aligned} i(t) \sim A & \left\{ [(x-y-K_d) + \sqrt{(x+y+K_d)^2 - 4xy}] G_p(t) + \right. \\ & [(y-x-K_d) + \sqrt{(x+y+K_d)^2 - 4xy}] G_L(t) + \\ & \left. [(x+y+K_d) - \sqrt{(x+y+K_d)^2 - 4xy}] G_C(t) \right\} \quad (vi) \end{aligned}$$

5 [00104] To obtain Kd , the following special cases for x and y can be used:

By adding the same amount of protein and ligand to the buffer (i.e. $x=y$)

$$\begin{aligned} i(t)|_{y=x} \sim A & \left\{ [(-K_d) + \sqrt{K_d^2 + 4xK_d}] G_p(t) + [(-K_d) + \sqrt{K_d^2 + 4xK_d}] G_L(t) + \right. \\ & \left. [(2x + K_d) - \sqrt{K_d^2 + 4xK_d}] G_C(t) \right\} \quad (vii1) \end{aligned}$$

[00105] By adding a fixed amount of protein and twice the amount of ligand to the buffer (i.e. $y=2x$):

$$\begin{aligned} i(t)|_{y=2x} \sim A & \left\{ [(-x-K_d) + \sqrt{(3x+K_d)^2 - 8x^2}] G_p(t) + \right. \\ & [(x-K_d) + \sqrt{(3x+K_d)^2 - 8x^2}] G_L(t) + \\ & \left. [(3x+K_d) - \sqrt{(3x+K_d)^2 - 8x^2}] G_C(t) \right\} \quad (vii2) \end{aligned}$$

[00106] By adding only protein to the buffer without ligand (i.e. $y=0$):

$$i(t)|_{y=0} \sim Ax G_p(t) \quad (viii1)$$

[00107] By adding only ligand to the buffer without protein (i.e. $x=0$):

$$i(t)|_{x=0} \sim Ay G_L(t) \quad (viii2)$$

15 [00108] As a result, the dissociation constant Kd can be obtained by solving Equations (vii), (viii). Also, the “temporal response” of protein, ligand, and protein-ligand complex, represented by $G_p(t)$, $G_L(t)$, and $G_C(t)$, respectively, can be obtained.

Example 3 CFD Computation

[00109] The transport of lysozyme solution inside a microchannel was modeled with the mass transfer equation (22), which included convection and diffusion, coupled with surface adsorption (23),

$$\frac{\partial C_A}{\partial t} = \nabla \cdot (D_{AB} \nabla C_A) - \mathbf{V} \cdot \nabla C_A \quad (22)$$

$$\frac{dC_A^*}{dt} = K_{ads} C_{AS} (C_{A(max)}^* - C_A^*) - K_{des} C_A^* \quad (23)$$

[00110] with lysozyme bulk concentration C_A , bulk solution concentration near the surface C_{AS} , surface adsorption concentration C_A^* , and maximum surface adsorption amount $C_{A(max)}^*$. Due to large protein-surface binding free energy, K_{des} was ignored in the computation to simplify the analysis. A fully developed laminar velocity profile was adopted. The microfluidic channel was initially filled with pure water before being flushed with protein solution of concentration C_{A0} . The governing equations, initial and boundary conditions in dimensional and scaled forms are shown below. COMSOL multiphysics software (Version 5.1, COMSOL Inc. USA) was used to solve the partial differential equations.

[00111] The mass transfer equation, which consists of convection and diffusion, coupled with the surface adsorption are simplified in 2-dimension as shown in FIG 12:

$$\frac{dC_A}{dt} + \vec{V}_x \frac{dC_A}{dx} = \frac{\partial}{\partial y^2} C_A \quad (ix)$$

[00112] Here, C_A is protein concentration, D_{AB} is protein diffusivity and the fully developed laminar velocity profile is $\vec{V}_x = 6U \frac{y}{h} (1 - \frac{y}{h})$ and U is the averaged velocity. The adsorption and desorption from biosensor surface are described by Langmuir adsorption model:

$$\frac{dC_A^*}{dt} = K_{Ads} C_{AS} (C_{A(max)}^* - C_A^*) - K_{Des} C_A^* \quad (x)$$

[00113] Where C_A^* is surface concentration, K_{Ads} and K_{Des} are adsorption and desorption rate constants, $C_{A(max)}^*$ is maximum surface concentration and $C_{AS} = C_A(y=0)$.

[00114] The BCs and ICs are the following:

$$\text{BC. 1: at } x = 0, C_A = C_{A0} \quad (xi)$$

$$\text{BC. 2: at } y = 0 \text{ and } x > 0, D_{AB} \frac{dC_A}{dy} = \frac{dC_A^*}{dt} = K_{Ads} C_{AS} (C_{A(max)}^* - C_A^*) - K_{Des} C_A^* \quad (xii)$$

$$\text{BC. 3: at } y = h \text{ and } x > 0, \frac{dC_A}{dy} = 0 \quad (\text{xiii})$$

$$\text{IC. 1: at } t = 0 \text{ and } x > 0, C_A = 0 \quad (\text{xiv})$$

$$\text{IC. 2: at } t = 0 \text{ and } x > 0, C_A^* = 0 \quad (\text{xv})$$

[00115] The dimensionless parameters listed in Table 5 are introduced to scale the equations (ix)-(xv):

Table 5 Definitions of Dimensionless Groups

Dimensionless Parameter	Definition	Dimensionless Parameter	Definition
$\theta_A = \frac{C_A}{C_{A0}}$	Concentration	$Pe = \frac{Uh}{D_{AB}}$	Péclet number
$\theta_A^* = \frac{C_A^*}{C_{A(\max)}^*}$	Surface concentration	$Da = \frac{K_{Ads} C_{A(\max)}^* h}{D_{AB}}$	Danköehler number
$\zeta = \frac{x}{h}$	Channel Length	$K_D = \frac{K_{Des}}{C_{A0} K_{Ads}}$	Equilibrium constant
$\eta = \frac{y}{h}$	Channel Height	$\varepsilon = \frac{C_{A0} h}{C_{A(\max)}^*}$	Relative concentration
$\tau = \frac{D_{AB} t}{h^2}$	Diffusion time		

[00116] The scaled form of governing equation is

$$\frac{d\theta_A}{d\zeta} + 6\eta(1-\eta)Pe \frac{d\theta_A}{d\zeta} = \frac{d^2\theta_A}{d\eta^2} \quad (\text{xvi})$$

10 [00117] The scaled form of the surface reaction is

$$\frac{d\theta_A^*}{d\tau} = \varepsilon Da [\theta_{As} \cdot (1 - \theta_A^*) - K_D \theta_A^*] \quad (\text{xvii})$$

[00118] The scaled form of the BCs and ICs are

$$\text{BC. 1: at } \zeta = 0, \theta_A = 1 \quad (\text{xviii})$$

$$\text{BC. 2: at } \eta = 0, \frac{d\theta_A}{d\eta} = \frac{1}{\varepsilon} \frac{d\theta_A^*}{d\tau} = Da [\theta_{As} \cdot (1 - \theta_A^*) - K_D \theta_A^*] \quad (\text{xix})$$

$$15 \quad \text{BC. 3: at } \eta = 1, \frac{d\theta_A}{d\eta} = 0 \quad (\text{xx})$$

$$\text{IC. 1: at } \tau = 0, \theta_A = 0 \quad (\text{xxi})$$

$$\text{IC. 2: at } \tau = 0, \theta_A^* = 0 \quad (\text{xxii})$$

Example 4 Atomistic Simulations

[00119] MD simulations were performed with Gromacs software package (version 4.6.5)⁶³ in NVT ensemble by using Charmm36 force field for protein and NAG molecules, and tip3p water model. Lysozyme crystal structure (pdb code: 3TXJ) was obtained from the Protein Data Bank and was with a net charge of +8e at pH 7 (see details below). The system was neutralized by adding Cl⁻ ions. In addition, 40 pairs of Na⁺ and Cl⁻ were added into the system to keep the ion concentration equal to 120 mM. To obtain solvation structures, pure lysozyme and lysozyme–NAG complex were first equilibrated in water environment for 50 ns. Detailed discussion about MD simulation is shown below.

[00120] Of the amino acids, arginine (Arg) and lysine (Lys) were protonated, glutamate (Glu) and aspartate (Asp) were deprotonated, and histidine (His) was treated neutral, resulting in a net charge of +8e at pH 7. The N and C termini remained uncapped. Time integration was performed with leap-frog algorithm using a time step of 1 fs. Berendsen thermostat at 298 K was used. The particle mesh Ewald summation was adopted to calculate long-range electrostatic interactions with a cut-off distance of 1.2 nm for the separation of the direct and reciprocal spaces. A spherical cut-off at 1.2 nm was imposed on Lennard-Jones interactions. The long-range dispersion effect was also calibrated.

[00121] A two-step procedure was adopted to predict protein adsorption and consequent surface polarization. First, hybrid molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) computations were performed to predict the protein initial orientation on Au (111) surface based on protein binding free energy, which consisted of protein–surface interactions and hydration or dehydration free energy, according to a previously established protocol to serve as an initial value for the following MD simulations. In MM/PBSA, the solvated protein and protein–NAG complex were treated as rigid bodies respectively, and were rotated around their center of mass on Au (111) surface (8.075 × 7.992 nm²) while fixing protein–surface minimum distances (i.e., 0.3 nm) to search for the most energetically favorable orientations. To simplify the computation in MM/PBSA, the nonpolarizable Au surface parameters were used for the Au–protein and Au–water interactions. The protein–surface distance was also changed to 0.26 nm, which was closer to the surface, and the same most top-ranking orientations for both pure protein and complex were identified from MM/PBSA. The surface tension of Au (111) ($\gamma = 1.41 \text{ J/m}^2$) was adopted from the literature report in MM/PBSA computations. Second, a full relaxation of the initial adsorbed lysozyme and lysozyme–NAG configurations was performed with full-atom MD simulations for 20 ns with polarizable force field parameters of Au (111) surfaces, which was developed by Walsh et al. and accounts for the interactions between peptides or

protein and the induced surface image charges by introducing dummy atoms to form rigid-rod dipoles free to rotate around atomic sites. Surface atoms were aligned with periodic boundary image atoms in accordance with the gold crystal lattice to mimic a large surface without boundary effects. Two repulsive walls were built on the top and bottom layers of the z-direction to confine solvent molecules. At the top of the gold surface, a water box of 7.6 nm height was built.

[00122] While this patent document contains many specifics, these should not be construed as limitations on the scope of any invention or of what may be claimed, but rather as descriptions of features that may be specific to particular embodiments of particular inventions. Certain features that are described in this patent document in the context of separate embodiments can also be implemented in combination in a single embodiment. Conversely, various features that are described in the context of a single embodiment can also be implemented in multiple embodiments separately or in any suitable subcombination. Moreover, although features may be described above as acting in certain combinations and even initially claimed as such, one or more features from a claimed combination can in some cases be excised from the combination, and the claimed combination may be directed to a subcombination or variation of a subcombination.

[00123] Similarly, while operations are depicted in the drawings in a particular order, this should not be understood as requiring that such operations be performed in the particular order shown or in sequential order, or that all illustrated operations be performed, to achieve desirable results. Moreover, the separation of various system components in the embodiments described in this patent document should not be understood as requiring such separation in all embodiments.

[00124] Only a few implementations and examples are described and other implementations, enhancements and variations can be made based on what is described and illustrated in this patent document.

CLAIMS

We claim:

1. A device for measuring a transient induced molecular electronic spectroscopy (TIMES) signal of a molecule, comprising:
 - 5 a microfluidic channel that can be filled with a solution;
 - a sensing pad comprising an electrode, wherein the electrode is in contact with the solution in the microfluidic channel;
 - a low-noise amplifier circuit;
 - at least one inlet coupled to a first end of the microfluidic channel;
 - 10 at least one outlet coupled to a second end of the microfluidic channel; and
 - a mechanism to control the flow rate of the solution in the microfluidic channel during the measurement.
2. The device of claim 1, wherein the microfluidic channel comprises a
15 polydimethylsiloxane (PDMS)-based material.
3. The device of claim 1 or 2, wherein the molecule comprises a protein, a ligand, or a protein-ligand complex.
4. The device of any one of claims 1-3, wherein the electrode is positioned to sense a diffusion flux of the molecule .
- 20 5. The device of claim 4, wherein the diffusion flux creates a concentration gradient along a height of the microfluidic channel, thereby producing a TIMES signal.
6. The device of any one of claims 1-5, comprising two inlets connected to the microfluidic channel, a first inlet to introduce a molecule, and a second inlet to introduce the solution.
- 25 7. The device of claim 6, wherein the solution is a buffer.
8. The device of any one of claims 1-7, wherein the low-noise amplifier is a transimpedance amplifier, and wherein the transimpedance is greater than 1 Mohm.
9. A method of detecting a molecule in a solution comprising:

conditioning the microfluidic channel of any one of claims 1-8;
allowing a solution containing the molecule to flow through a first inlet connected to the microfluidic channel;
receiving a signal generated by the molecule in contact with the electrode in the
5 microfluidic channel;
performing an analog-to-digital conversion of the signal to generate a digitized signal;
and
processing the digitized signal by a processor to obtain the transient induced molecular electronic spectroscopy (TIMES) dissociation constant of the molecule.

10 10. The method of claim 9, wherein conditioning the microfluidic channel comprises:
injecting the solution through the first inlet and a second inlet to flush the microfluidic channel;
injecting the solution containing the molecule through the first inlet;
15 stopping the injection of the first inlet;
injecting the solution through the second inlet to wash the microfluidic channel, such that a liquid interface between the solution containing the molecule and the solution forms near the input of the microfluidic channel.

20 11. The method of claim 9 or claim 10, wherein the molecule is a protein, a ligand, or a protein-ligand complex.

12. The method of any one of claims 9-11, wherein the solution is a buffer.

25 13. A method of detecting the binding of a first molecule and a second molecule comprising:
detecting the first molecule according to claim 9 or claim 10;
detecting the second molecule according to claim 9 or claim 10;
mixing the first molecule and the second molecule in a solution such that a complex is
30 formed between the two molecules;
detecting the complex of the first molecule and the second molecule according to claim 9 and claim 10;

comparing the TIMES dissociation constants of the first molecule, the second molecule and the complex of the first molecule and the second molecule to determine the binding of the first molecule and the second molecule.

5 14. The method of claim 13, wherein the first molecule and the second molecule are a protein and a ligand, respectively.

15. The method of claim 13, wherein different ratios of the first molecule and the second molecule are mixed to form the complex.

10

16. The method of claim 13, wherein the difference in a molecular weight and chemical composition between the first molecule and the complex of the first molecule and the second molecule is less than 1%.

15 17. The method of claim The method of claim 13, wherein the difference in a molecular weight and chemical composition between the second molecule and the complex of the first molecule and the second molecule is less than 1%.

18. The method of claim 13, wherein the detection of the binding of the first molecule and the second molecule is performed without any modification, labeling, or immobilization of the first molecule or the second molecule.

20

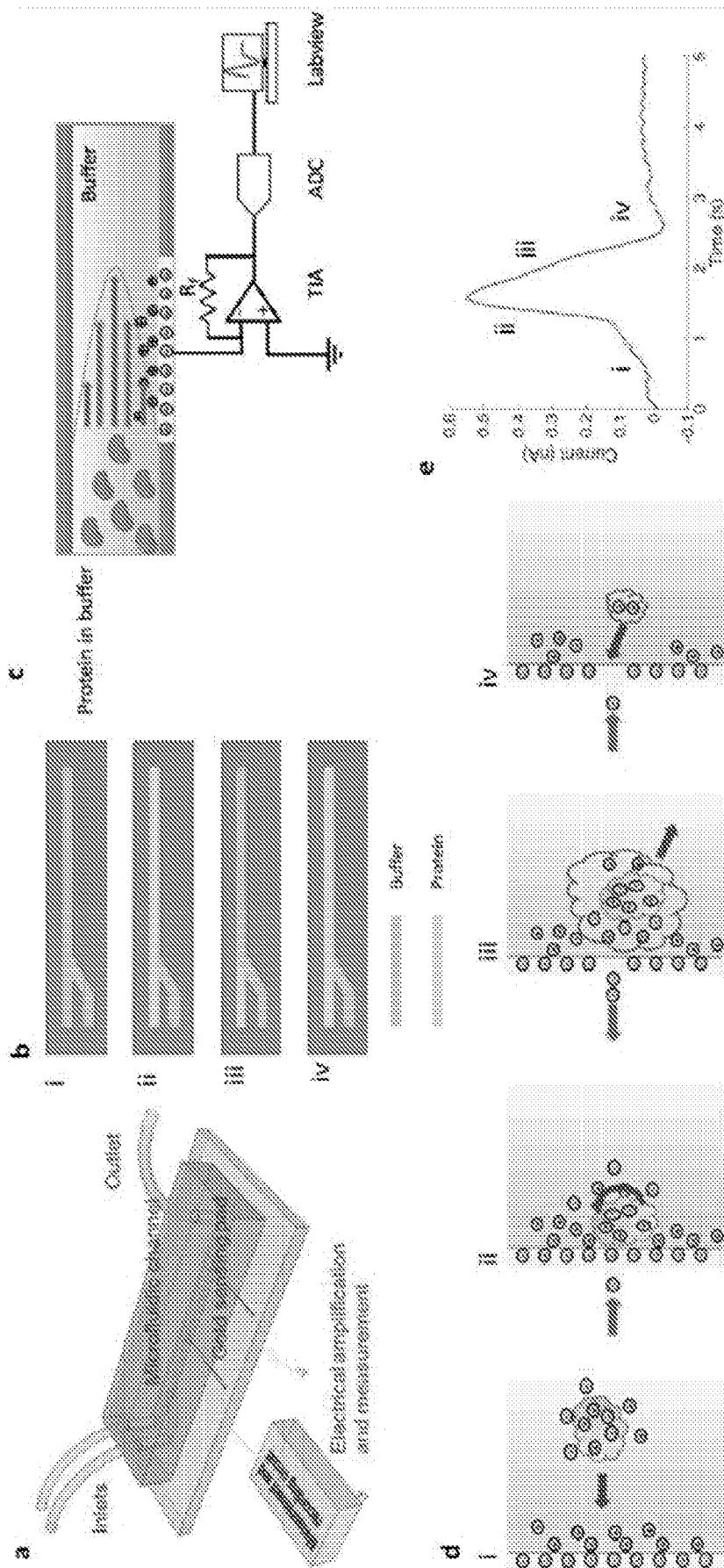


FIG. 1

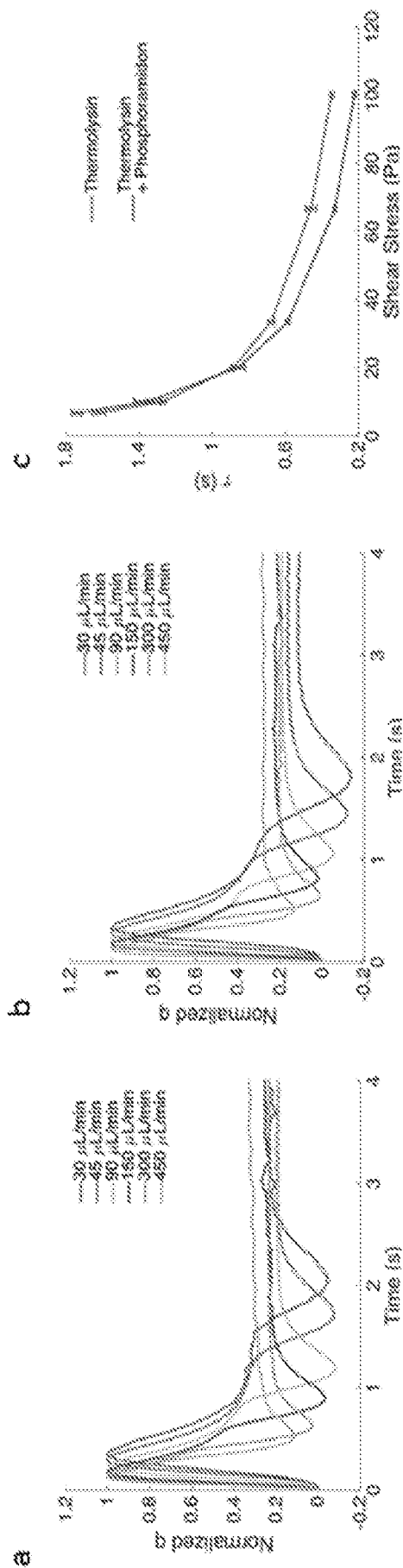


FIG. 2

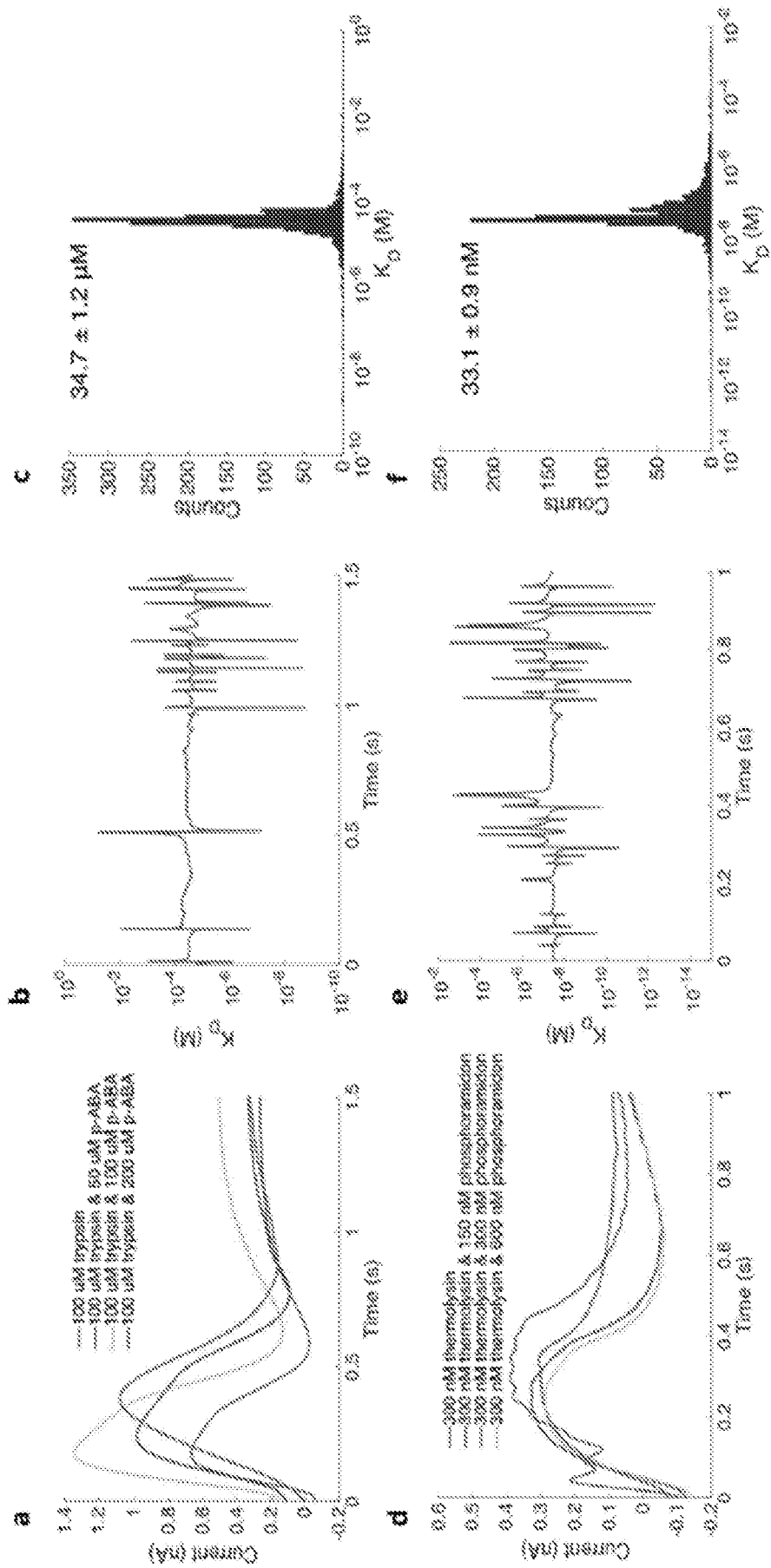


FIG. 3

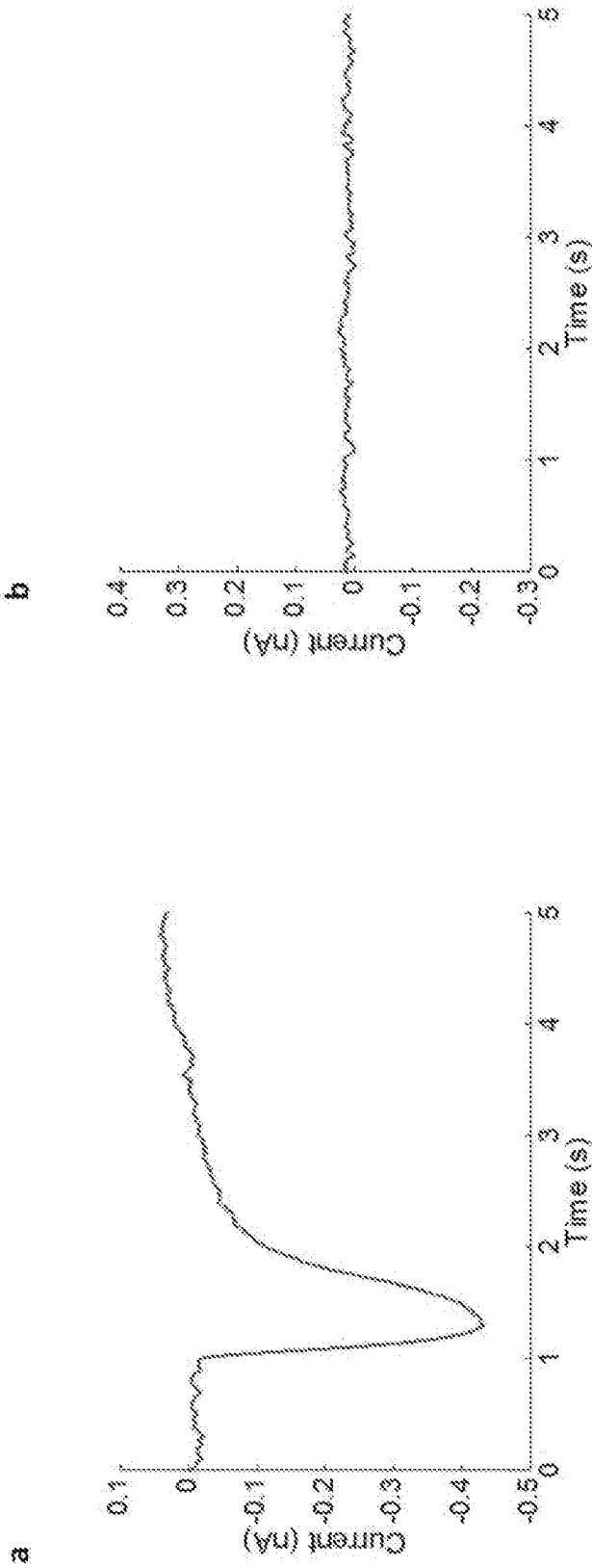


FIG. 4

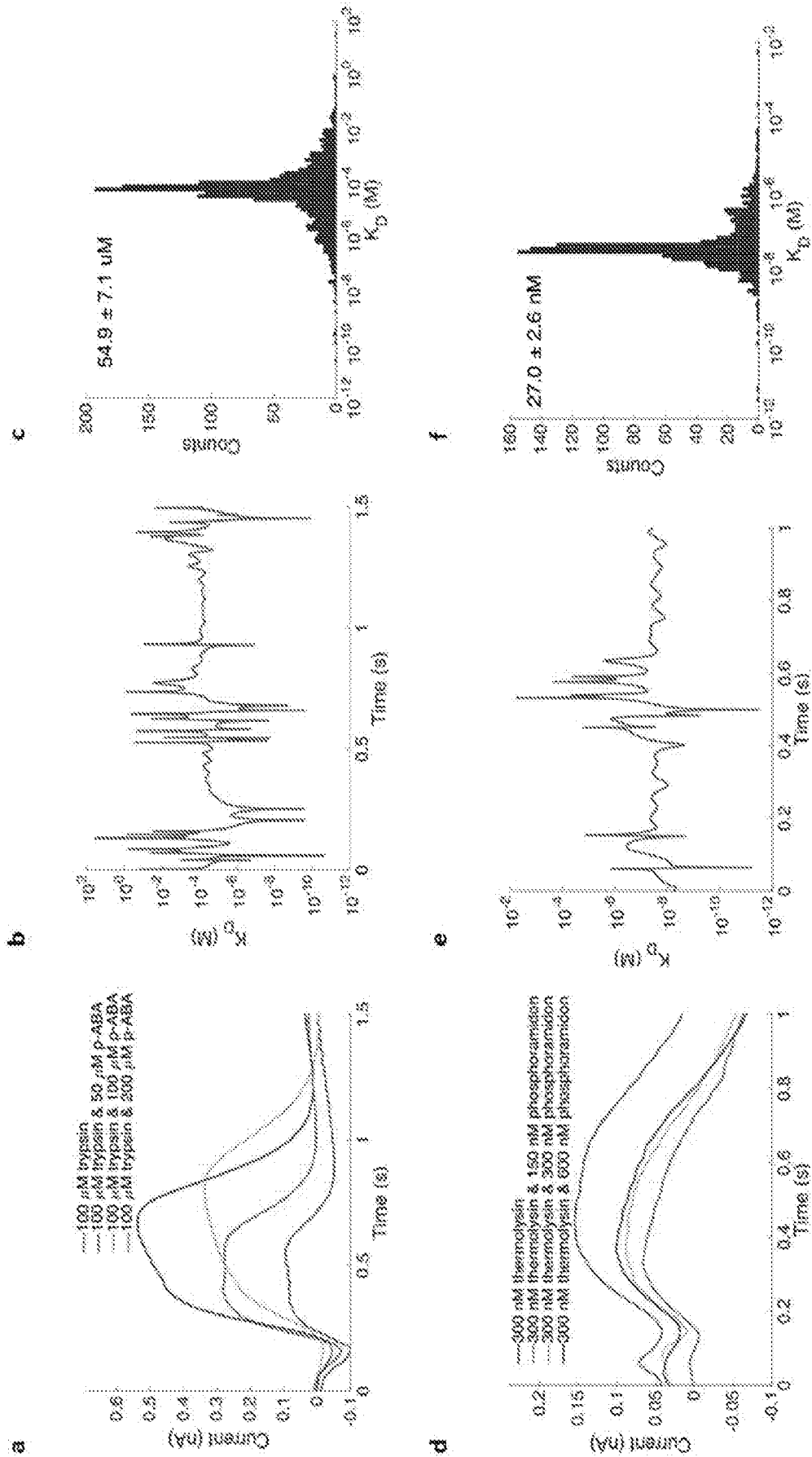


FIG. 5

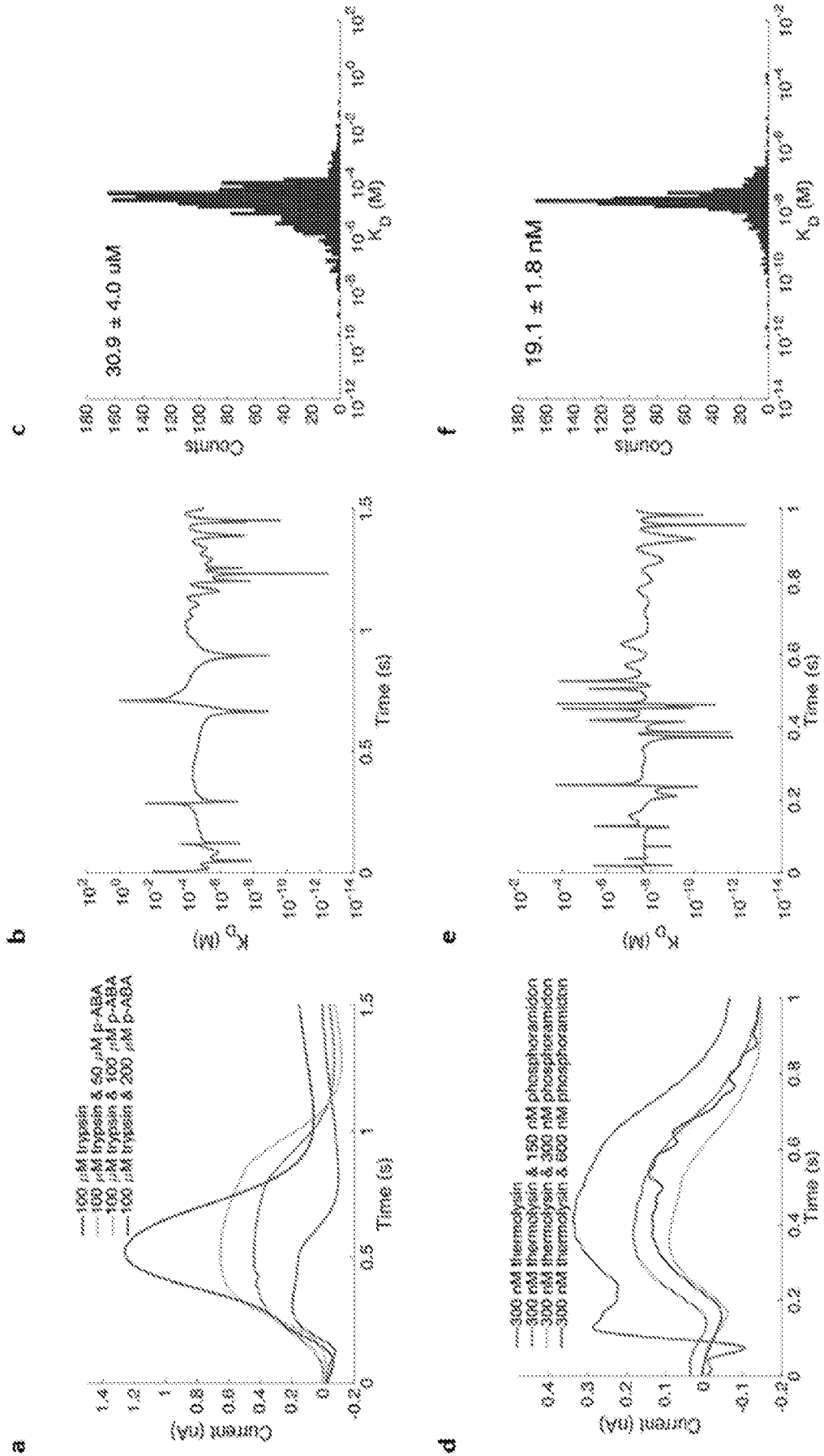


FIG. 6

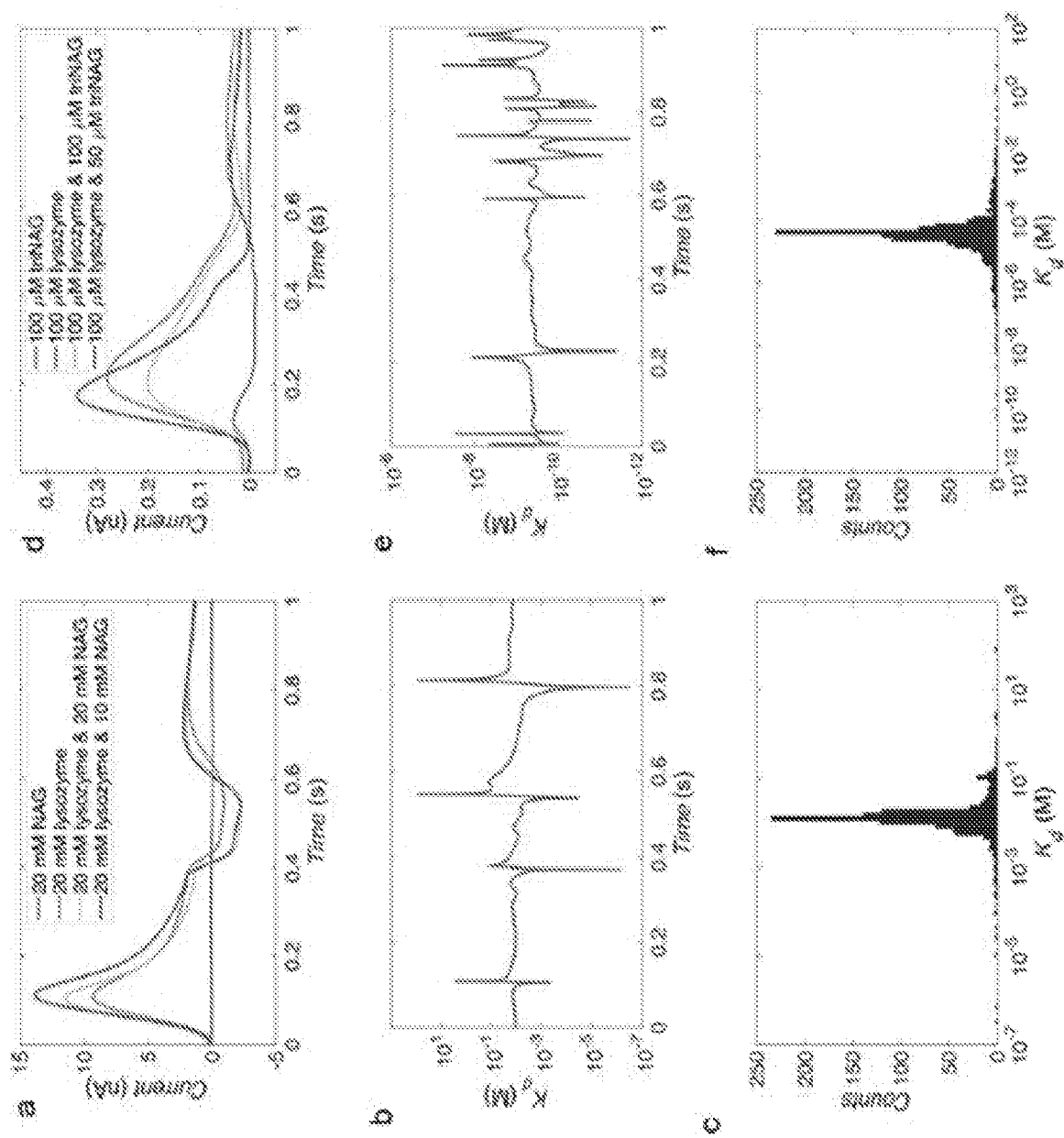


FIG. 7

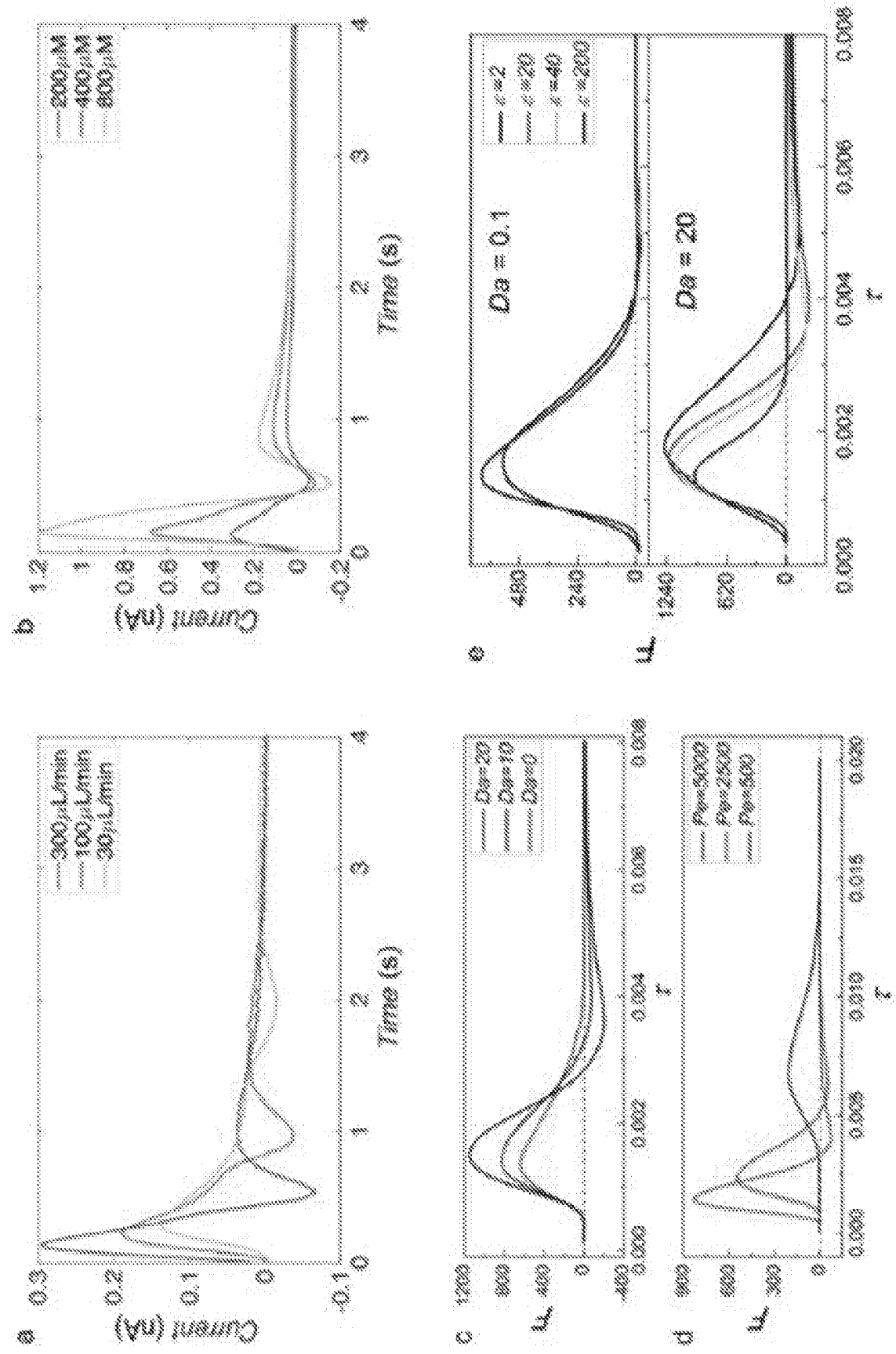


FIG. 8

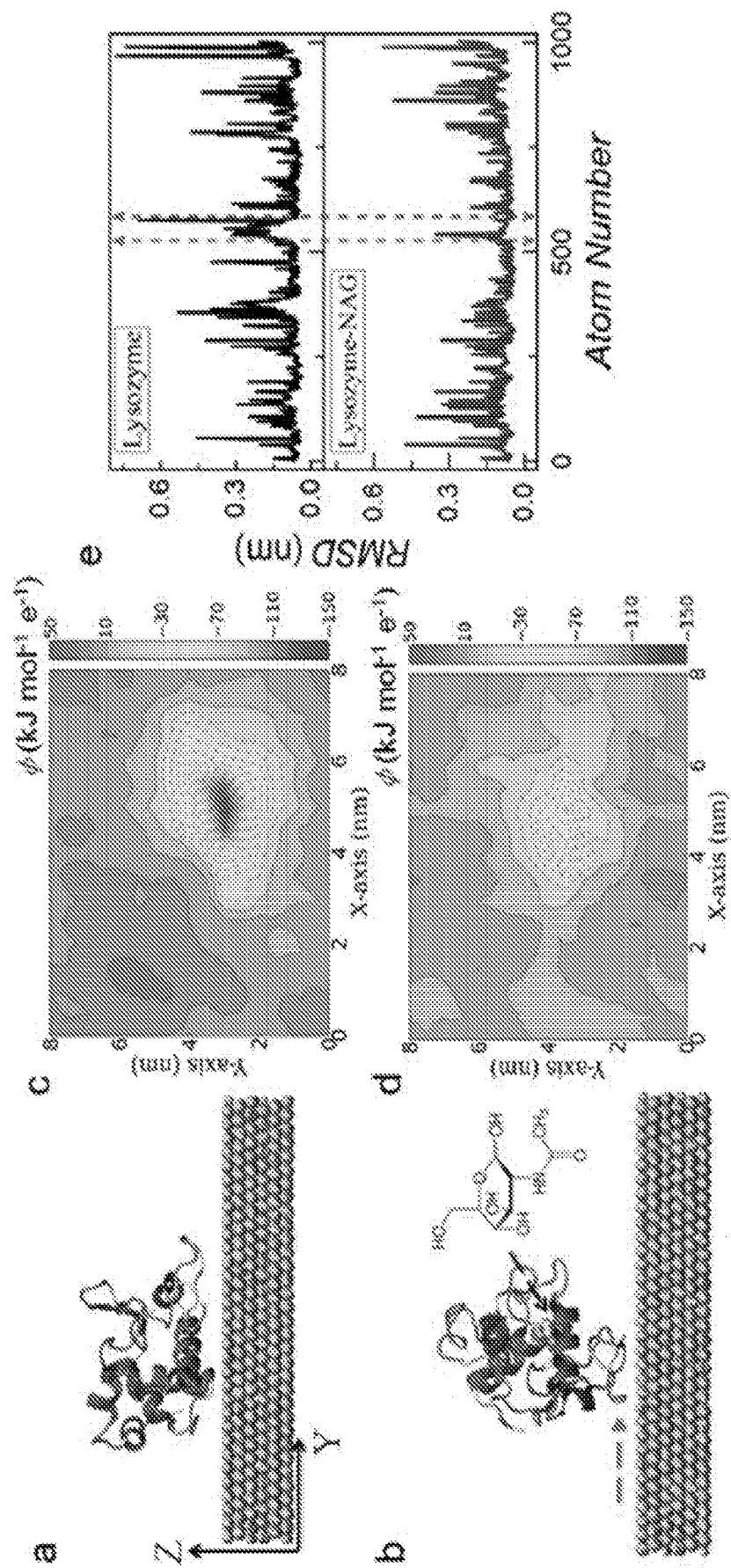


FIG. 9

10/12

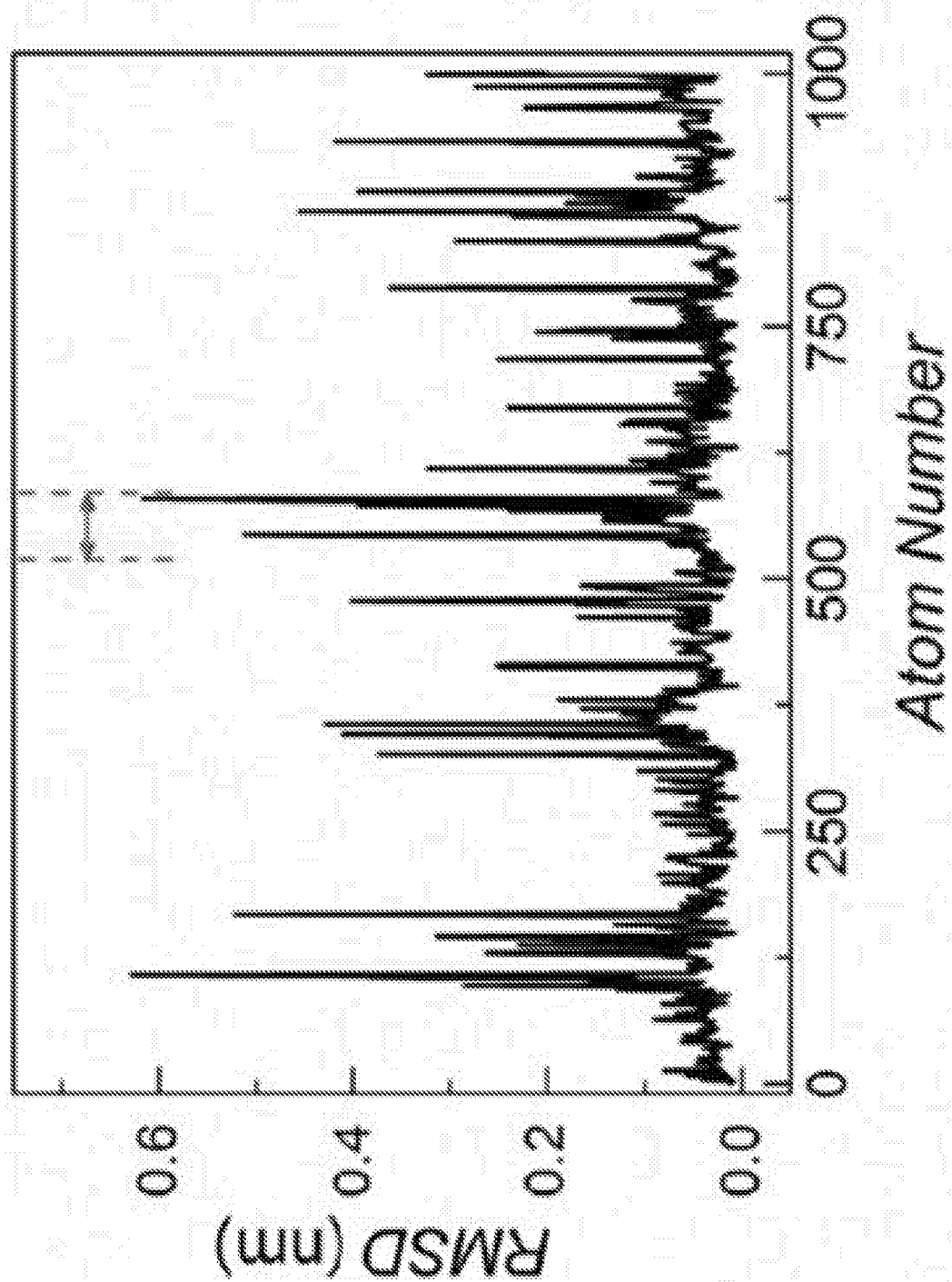


FIG. 10

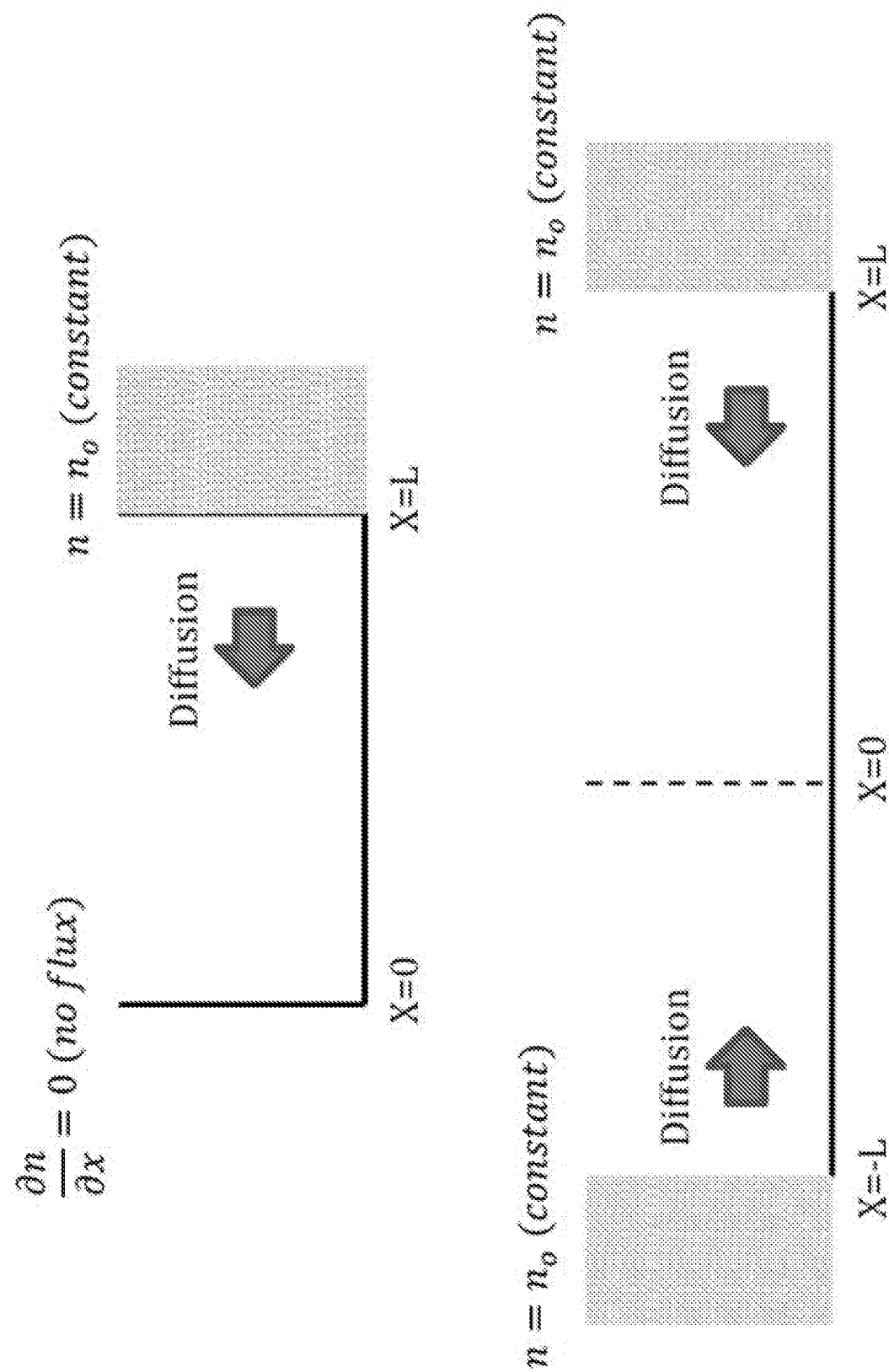


FIG. 11

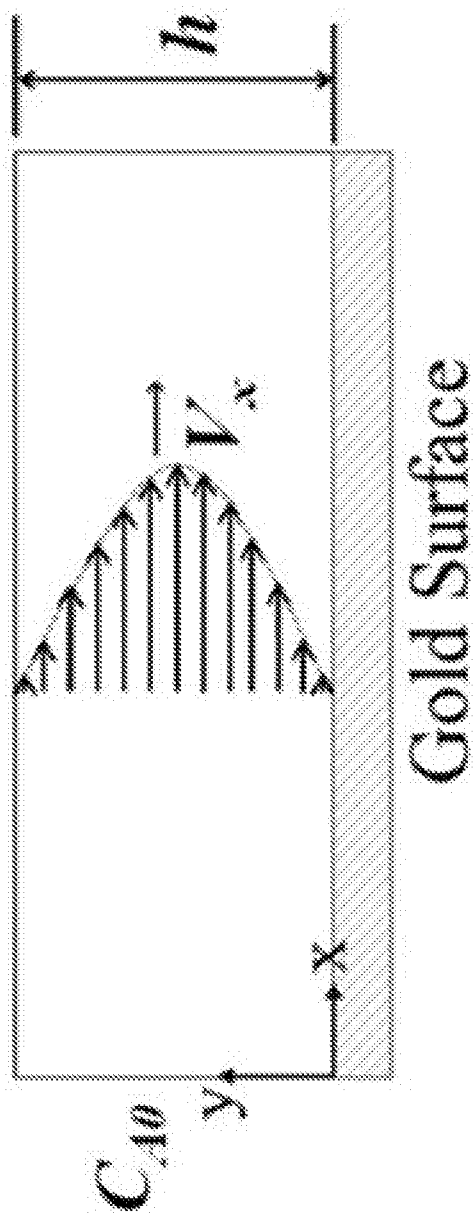


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/21486

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 21/64 (2017.01)

CPC - G01N 21/6428, G01N 2021/6441, G01N 21/6408, G01N 2021/6432

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2008/0274493 A1 (Quake et al.) 06 November 2008 (06.11.2008), abstract; FIG. 2; para [0170], [0202], [0291], claim 1	1-3
A	US 2012/0040370 A1 (Orwar et al.) 16 February 2012 (16.02.2012), paras [0024], [0034], [0120]; claims 1, 24	1-3
A	US 2009/0011430 A1 (Ateya et al.) 08 January 2009 (08.01.2009), abstract; para [0037], [0065]	1-3
A, P	ZHANG et al., Transient Induced Molecular Electronic Spectroscopy (TIMES) for study of protein-ligand interactions, Scientific Reports, 6:35570, October 19, 2016, entire document	1-3
A, P	US 2016/0252517 A1 (Lo et al.) 01 September 2016 (01.09.2016), entire document	1-3

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

29 April 2017

Date of mailing of the international search report

30 MAY 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/21486

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.