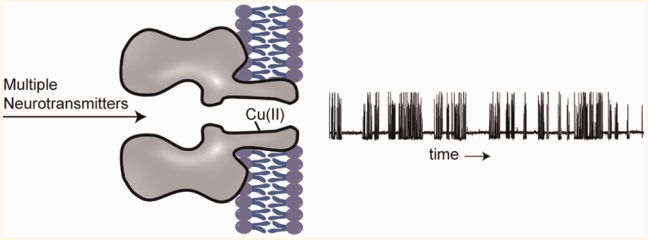
Real-Time Stochastic Detection of Multiple Neurotransmitters with a Protein Nanopore

Arnold J. Boersma,[†] Keith L. Brain,[‡] and Hagan Bayley[†,*]

[†]Department of Chemistry, University of Oxford, Oxford, OX1 3TA, United Kingdom and [‡]School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

ABSTRACT



The detection of several different neurotransmitters with the same sensor in real-time would be a powerful asset to the field of neurochemistry. We have developed a detector for a broad range of neurotransmitters including amino acids, catecholamines, and nucleotides, which relies on the reversible binding of the analytes to a copper(II) complex within an engineered protein nanopore.

KEYWORDS: neurotransmitters · α-hemolysin · nanopore · single-molecule chemistry · stochastic sensing · biosensor

Neurochemical measurements are a bottleneck in understanding the physiological manifestations of chemical neurotransmission.[1] Frequently, multiple neurotransmitters and neuromodulators are released into the synaptic cleft from the same presynaptic neuron. For example, ATP is co-released as a neurotransmitter or neuromodulator at almost every central and peripheral synapse.[2] This process and its outcomes are poorly understood and real-time techniques for the simultaneous detection of multiple neurotransmitters would be valuable in this regard.[3] Carbon electrodes have been widely applied for the electrochemical determination of dopamine[4] and other neurotransmitters,[5] while a mixture of catecholamines can be determined using fast scanning cyclic voltammetry.[6] In general, only one neurotransmitter is monitored at a time, and there is often a strong background signal from irrelevant oxidizable compounds. Techniques for the observation of multiple neurotransmitters often use sampling techniques, such as dialysis, followed by HPLC or capillary electrophoresis.[1,3] The fastest reported sampling intervals are ~14 s.[7]

The protein nanopore α-hemolysin (αHL) has been widely employed for the stochastic detection of various analytes.[8,9] A single αHL nanopore is inserted in a lipid bilayer, and ions flow through the pore upon application of a potential. The reversible binding of molecules at sites engineered within the pore, observed as current blockades, allows the quantification of an analyte based on the number of binding events per second. The magnitude of the current blockade and the mean dwell time of an analyte within the pore can be used for analyte identification. Because events arising from related analytes binding to the same promiscuous site are separated in the time domain by a single-molecule detector, several different analytes can be determined simultaneously.[10,11]

The transmembrane β barrel of the αHL pore lends itself well for the engineering of analyte binding sites.[12] Successful strategies include the employment of (i) amino acid residues with side-chains that interact with analytes,[11,13,14] (ii) metal chelating groups,[15] (iii) noncovalently attached adapters, such as cyclodextrins lodged inside the pore,[16,17] and (iv) amino acids with reactive side chains, such as cysteine residues.[18,19] By using these approaches, a wide variety of analytes have been detected, including small molecules, divalent metal ions, peptides, and polynucleotides.[8,9] Here, we present a method to detect analytes with the αHL nanopore, which allows the determination of multiple neurotransmitters simultaneously in real-time (Figure 1). We make use of the fact that many neurotransmitters bind metal ions, especially copper(II), in a reversible manner.

* Address correspondence to hagan.bayley@chem.ox.ac.uk.

Received for review March 14, 2012 and accepted May 22, 2012.

Published online May 22, 2012
10.1021/nn301125y

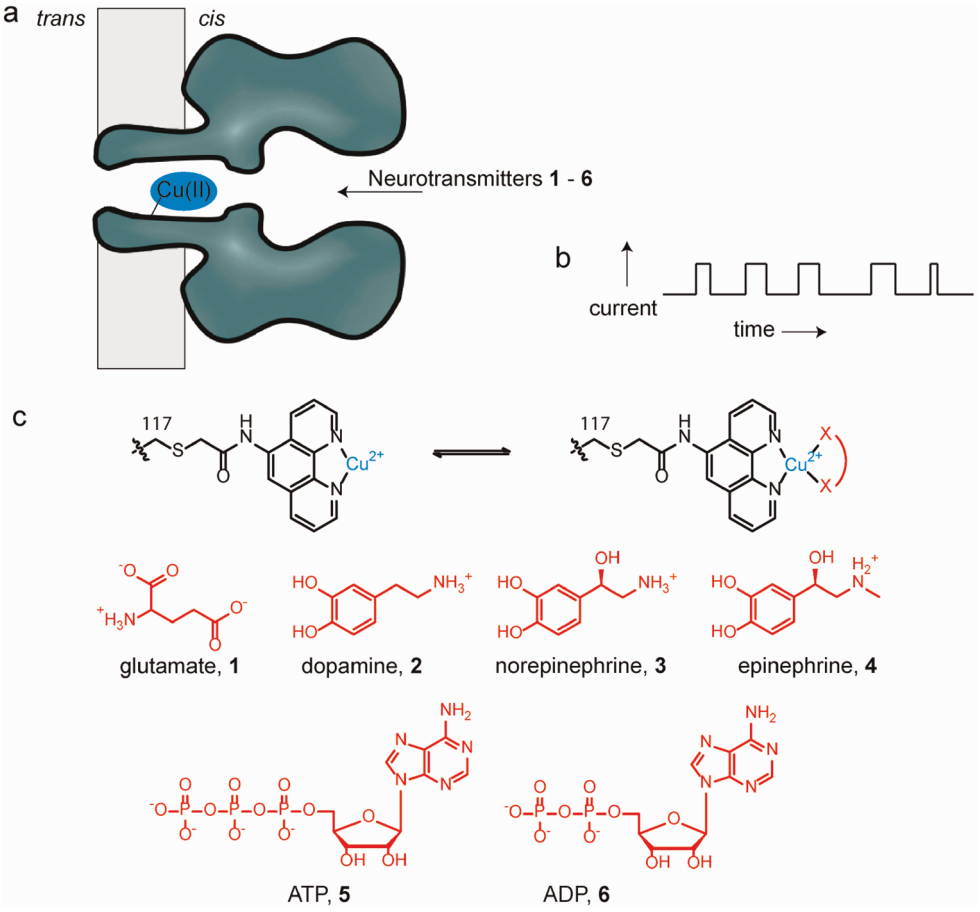


Figure 1. Detection of copper(II)-chelating neurotransmitters. (a) Detection concept. An engineered αHL nanopore is inserted into the bilayer from the cis (grounded) compartment, which contains Krebs' buffer (118 mM NaCl, 10 mM MOPS, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 11.1 mM D-glucose, pH 7.4). Copper(II) is added to the trans side of the bilayer and binds the αHL pore at the covalently attached phenanthroline. (b) Analytes presented from the cis side bind reversibly to the copper(II) complex resulting in current blocks. (c) Structures of neurotransmitters that bind the copper(II)−phenanthroline complex.

RESULTS AND DISCUSSION

Copper(II) Binding. A copper(II) ion was bound within an engineered αHL pore modified with a phenanthroline derivative. Phenanthroline has a high affinity for copper(II) but leaves sites on the metal ion available for the coordination of neurotransmitters. In the present case, neurotransmitters capable of bidentate binding to copper(II) were observed, as they can compete with water for copper(II) coordination. Phenanthroline was installed by post-translational chemical modification of an αHL monomer with a cysteine at position 117. Assembly of the modified monomer with six cysteine-free wild-type monomers formed a heteroheptameric nanopore containing a single phenanthroline within the transmembrane β barrel. The pore was inserted into a planar lipid bilayer with Krebs' buffer on the cis side (Figure 1a). The trans compartment contained a buffer with a higher concentration of ions (0.5 M NaCl) to increase the current flow through the pore, which passed -12 ± 1 pA at -50 mV under these conditions. The addition of 20 μM copper(II) chloride to the trans side increased the current by 1.89 ± 0.09 pA ($n = 4$). The current level seen in the absence of copper(II) was no longer observed after the addition of copper(II), indicating strong binding of the metal ion (Supporting Information).

Glutamate Detection. The addition of glutamate **1**, which coordinates copper(II) through the amine nitrogen and a carboxyl oxygen,[20] resulted in reversible current blockades of 2.7 ± 0.3 pA, with a mean lifetime of 290 ± 60 ms (Figure 2a). A scatter plot of current block duration *versus* amplitude shows that glutamate yields a single population of events (Supporting Information). The rates associated with the binding events were determined by analyzing idealized current traces with QuB software (www.qub.buffalo.edu) (Table 1). As expected for a bimolecular interaction between glutamate and copper(II)-phenanthroline,[20] the association rate shows a first-order dependence on the concentration of glutamate (Supporting Information), while dissociation is independent of the concentration.

Neurotransmitter Binding. The additional neurotransmitters **2**−**6** were also tested with the engineered αHL

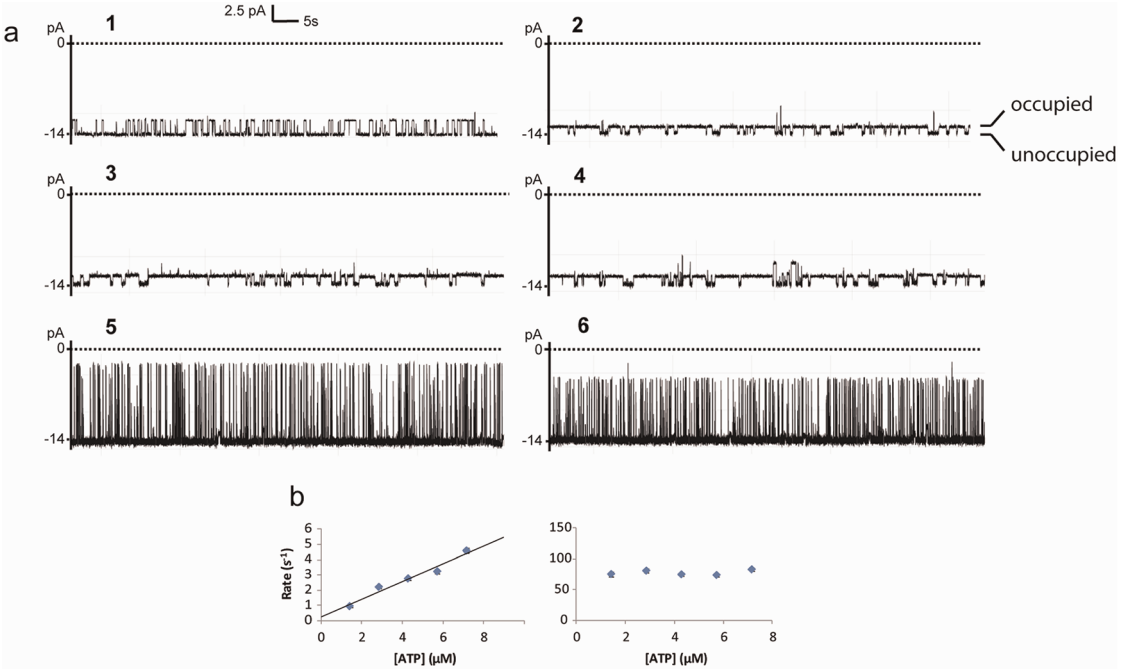


Figure 2. Detection of six neurotransmitters using the Cu(II)−phenanthroline-modified αHL pore. (a) Current recordings for each neurotransmitter are presented. Concentrations: 1, 5 μM; 2, 2 μM; 3, 1.5 μM; 4, 1.5 μM; 5, 7 μM; 6, 5 μM. All traces are displayed on the same scale. The amino acid 1, the catecholamines 2−4, and the nucleotides 5 and 6 display significantly different current block amplitudes and mean dwell times. (b) Representative titration with ATP, showing a first order dependence on ATP concentration for association, and the concentration independence of dissociation (linear fit, $R^2 = 0.96$). Conditions: cis (grounded) side, Krebs' buffer (see Figure 1); trans side, 0.5 M NaCl, 10 mM MOPS, 20 μM $CuCl_2$, pH 7.4, at an applied potential of −50 mV.

nanopore and in all cases gave reversible blocks with scatter plots showing single populations of events (Supporting Information). Again, first-order dependencies on the analyte concentration were observed for association, while dissociation was concentration independent (Figure 2b). However, the association and dissociation rate constants differ significantly between the neurotransmitters. The catecholamines **2**−**4** bind most strongly to the copper(II) with mean lifetimes of about a second. Because the pore is occupied for around a second, multiple pores might be employed to increase the frequency of observed events when fast detection of changes in neurotransmitter concentration is required.[21] Alternatively, the mean dwell time of the analyte might be reduced by using alternative metal ions or chelators other than phenanthroline, or by adjusting the pH or applied potential. The positively charged analytes, dopamine, epinephrine, and norepinephrine (**2**−**4**) have the highest k_{on} values, which may be enhanced by the negative applied potential.[22] The amplitude of current block depends roughly on the size of the analyte; the most bulky, ATP and ADP, **5** and **6**, give the largest current block. However, the smaller glutamate gives a higher block than the catecholamines. The current block is therefore likely to be a combination of multiple factors, for example, the hydrodynamic radius of the bound complex, ionic interactions with buffer components such as magnesium(II), and the charge of the analyte.

TABLE 1. Binding Characteristics of Neurotransmitters 1−6 to the Cu(II)phenanthroline-Modified αHL Pore[a]

Nt	block (pA)	τ_{off} (ms)	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_a (μM^{-1})
1	2.7 ± 0.3	290 ± 60	0.23 ± 0.04	3.6 ± 0.7	0.064 ± 0.011
2	0.90 ± 0.10	1100 ± 200	1.33 ± 0.06	1.0 ± 0.2	1.4 ± 0.2
3	1.1 ± 0.2	1090 ± 90	1.6 ± 0.2	0.92 ± 0.08	1.7 ± 0.1
4	0.94 ± 0.15	1600 ± 300	1.7 ± 0.2	0.66 ± 0.14	2.5 ± 0.2
5	9.1 ± 1.7	32 ± 6	0.47 ± 0.15	32 ± 5	0.016 ± 0.002
6	7.4 ± 0.9	18 ± 5	0.88 ± 0.27	59 ± 17	0.016 ± 0.005

[a] Conditions as in Figure 2. Nt = neurotransmitter. Errors represent the standard deviation of the outcomes of three 5-point titrations of the analyte, each with different pores. The mean current block was determined after fitting an all-points histogram of the amplitudes in each experimental single-channel trace to a Gaussian. The mean dwell times τ_{off} were determined by fitting histograms of events from each idealized experimental single-channel trace to an exponential function. The dwell times are independent of analyte concentration. The mean k_{on} and k_{off} values were determined by modeling the idealized experimental single channel traces with QuB, and subsequently plotting the observed rates *versus* the concentration of analyte. The slope of the association rate *versus* concentration yielded k_{on}, while k_{off} values were obtained by averaging the dissociation rates. The association rate constant k_{on} and the dissociation rate constant k_{off} are systematically affected by the applied potential.[22−24] All measurements reported here have been performed at an applied potential of −50 mV. Equilibrium association constants K_a were obtained by dividing k_{on} by k_{off}.

Multineurotransmitter Detection. The fact that neurotransmitters can be distinguished by their mean dwell times and current block amplitudes permits

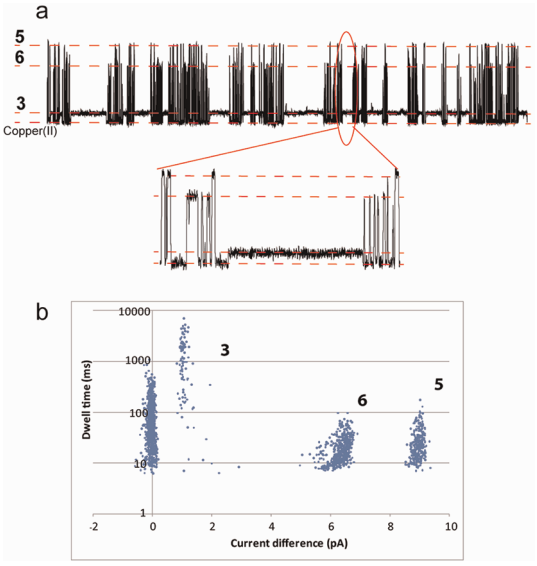


Figure 3. Simultaneous detection of multiple neurotransmitters. (a) A one-minute recording is displayed. Conditions as in Figure 2. Norepinephrine 3, 1 μM; ATP 5, 5 μM; ADP 6, 5 μM. The expansion is a trace of two seconds. (b) Scatter plot showing dwell times *versus* current block for individual analyte binding events.

the simultaneous examination of multiple neurotransmitters. To demonstrate the potential of the sensor, the principle neurotransmitters secreted from a sympathetic nerve terminal, that is, ATP, ADP, and norepinephrine,[25] were tested together. The three transmitters can clearly be distinguished by visual inspection of a current recording (Figure 3a). In a scatter plot of dwell times *versus* current block, three clearly separated populations are apparent (Figure 3b).

CONCLUSIONS

We have demonstrated the binding of six important neurotransmitters to a Cu(II)phenanthroline-modified αHL nanopore, and we are able to distinguish different classes of neurotransmitter, that is, amino acids, catecholamines, and nucleotides. This method holds considerable potential for use in *ex vivo* (*i.e.,* brain slices) and *in vitro* (*i.e.,* cell culture) measurements. The approach might be combined with recent innovations in bilayer technology such as stable bilayers on glass pipettes and polymerized bilayers.[26,27] Furthermore, bilayers have been made as small as 200 nm in diameter,[26] which will allow excellent spatial resolution. By this means, nanopore measurements could provide a time-dependent fingerprint of secreted neurotransmitters, for example in response to the application of drugs to tissues. Simultaneous, local monitoring of multiple neurotransmitters with single-molecule resolution, has the potential to transform the study of neurotransmitter costorage and cotransmission, including the functional investigation of the vesicular neurotransmitter transporters, such as the recently identified ATP transporter VMAT.[28] One key outcome could be the development of new pharmacological tools to differentially regulate neurotransmitter storage and hence modulate nerve function as it begins to fail in diseases as diverse as diabetes (through autonomic neuropathy) and the dopaminergic failure of Parkinson's disease.

METHODS

Preparation of αHL Monomers. The [^{35}S]methionine-labeled polypeptide αHL T117C-D8 was prepared by *in vitro* transcription and translation (IVTT).[29] The translation mix was subjected to gel filtration in 200 mM Tris.HCl, pH 8.0, on a Micro Bio-Spin 6 gel filtration column (Bio-Rad), and then *N*-(1,10-phenanthrolin-5-yl)iodoacetamide (Phen-IA) (100 mM stock solution in DMF, final concentration 10 mM) was added to the αHL monomers in Tris.HCl buffer, pH 8.0 (100 μL). The solution was mixed by pipetting, left for 1 h at room temperature, and then passed over two Micro Bio-Spin 6 gel filtration columns, to remove the unreacted Phen-IA. A portion (2 μL) was mixed with XT sample buffer (8 μL) and heated at 94 °C for 10 min, before analysis by electrophoresis in a 12% SDS-polyacrylamide gel (Criterion XT Bis-Tris gel, Bio-Rad) at 200 V for 50 min with XT MOPS running buffer (Bio-Rad). The gel was dried under vacuum, and the protein bands were visualized by autoradiography. The remainder of the reaction mix was stored at −80 °C.

Preparation of αHL Heteroheptamers. The chemically modified αHL T117C-D8 monomer (110 μL) was mixed with WT (wild-type) αHL polypeptide (50 μL, prepared by IVTT). A suspension of rabbit red blood cell membranes (3 μL, 4.2 mg membrane protein mL^{-1}) was washed twice with MBSA buffer (10 mM MOPS, 150 mM NaCl, 1 mg mL^{-1} bovine serum albumin, pH 7.4, titrated with HCl, 500 μL). The washed membranes were resuspended with the protein mixture. After 1 h at 37 °C, the membranes were pelleted by centrifugation for 5 min at 21 000*g*, resuspended in MBSA (500 μL) and recovered again by centrifugation. The membrane pellet was solubilized in Laemmli sample buffer (62.5 mM Tris.HCl, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, 30 μL) without heating and loaded in one lane of a 5% SDS polyacrylamide gel. The gel was run in TGS running buffer (25 mM Tris.HCl, 192 mM glycine, 0.1% SDS) at 50 V overnight. The gel was then dried under vacuum without heating onto Whatman 3MM paper and exposed to X-ray film overnight (Supporting Information, Figure S2). The band that corresponded to the $(WT)_6(Mutant\text{-}D8)_1$ heteroheptamer was excised. The gel was hydrated in MOPS buffer (300 μL, 10 mM MOPS, pH 7.0) for 30 min, and the paper was removed. The excised gel was then crushed with a plastic pestle, and the resulting suspension was incubated at room temperature for 30 min. The material was filtered through a 0.2 μm cellulose acetate filter (Rainin) at 16 000*g* for 30 min. The filtrate was aliquoted and stored at −80 °C.

Planar Lipid Bilayer Recordings. Single-channel recordings were carried out by using folded planar lipid bilayers, as described previously.[13] The cis chamber contained 3 mL of Krebs' buffer (118 mM NaCl, 10 mM MOPS, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 11.1 mM D-glucose, pH 7.4). The Krebs' solution was buffered by MOPS instead of the more commonly used phosphate because phosphate was expected to coordinate to copper(II). The trans chamber contained 3 mL of 500 mM NaCl, 10 mM MOPS, pH 7.4. The protein was added to the grounded cis chamber. A potential difference of −50 mV was applied through Ag/AgCl electrodes, which were set in 2% agarose containing 3 M NaCl. The negative potential indicates a higher negative potential in the trans chamber. $CuCl_2$ (3 μL, 20 mM in

water) was added to the trans chamber after a single pore had inserted to give a final concentration of 20 μM. The neurotransmitters (as ~1000× stock solutions in water or buffer) were added to the cis chamber, which was mixed with a pipet prior to measurement. The single-channel current was amplified by using a patch-clamp amplifier (Axopatch 200B, Axon Instruments), filtered with a low-pass Bessel filter (80 dB/decade) with a corner frequency of 1 kHz, and then digitized with a Digidata 1320 A/D converter (Axon Instruments) at a sampling frequency of 5 kHz, giving a time resolution of about 200 μs. The acquisition software was Clampex 10.2 (Molecular Devices).

Data Analysis. All current traces were filtered digitally with a 200 Hz (in the cases of ATP and ADP) or 50 Hz Bessel (8-pole) filter in Clampfit 10.2 (Molecular Devices). A single-channel search was performed to identify the unoccupied and partially blocked states. The association and dissociation rate constants for neurotransmitter binding to the Cu^{2+} center in the lumen of the pore were calculated by analysis of idealized single-channel recording traces, obtained in Clampfit, with the software QuB (State University of New York at Buffalo). Briefly, the program derives the lifetimes and frequency of occurrence of all states (no Cu^{2+} bound, Cu^{2+} bound, and neurotransmitter bound) from the idealized trace. The observed association and dissociation rates were plotted *versus* the concentration of neurotransmitter. The binding of each neurotransmitter to the pore was first order in neurotransmitter, and the rate constants were extracted from the slopes of the plots. The dissociation of the neurotransmitters from the Cu(II)−phenanthroline was independent of the concentrations of neurotransmitter, and the rate constants were obtained by averaging the dissociation rates (s^{-1}) obtained at different concentrations of neurotransmitter.

Conflict of Interest: The authors declare the following competing financial interest(s): H.B. is the Founder, a Director and a share-holder of Oxford Nanopore Technologies.

Acknowledgment. This work was supported by The Netherlands Organization for Scientific Research (NWO, Rubicon Fellowship, A.J.B.), the Medical Research Council, and Oxford Nanopore Technologies.

Supporting Information Available: Experimental procedures; copper(II) binding studies; scatter plots and kinetics of neurotransmitter binding. This material is available free of charge *via* the Internet at http://pubs.acs.org.

Note Added after ASAP Publication: Reference 6 was incorrect in the version published online May 22, 2012. The correct ref 6 was reposted with the paper June 5, 2012.

REFERENCES AND NOTES

1. Robinson, D. L.; Hermans, A.; Seipel, A. T.; Wightman, R. M. Monitoring Rapid Chemical Communication in the Brain. *Chem. Rev.* **2008**, *108*, 2554–2584.
2. Burnstock, G. Physiology and Pathophysiology of Purinergic Neurotransmission. *Physiol. Rev.* **2007**, *87*, 659–797.
3. Perry, M.; Li, Q.; Kennedy, R. T. Review of Recent Advances in Analytical Techniques for the Determination of Neurotransmitters. *Anal. Chim. Acta* **2009**, *653*, 1–22.
4. Wightman, R. M. Probing Cellular Chemistry in Biological Systems with Microelectrodes. *Science* **2006**, *311*, 1570–1574.
5. Kachoosangi, R. T.; Compton, R. G. A Simple Electroanalytical Methodology for the Simultaneous Determination of Dopamine, Serotonin and Ascorbic Acid Using an Unmodified Edge Plane Pyrolytic Graphite Electrode. *Anal. Bioanal. Chem.* **2007**, *387*, 2793–2800.
6. Threlfell, S.; Cragg, S. J. *Electrochemical Methods for Neuroscience*; Michael, A. C., Borland, L. M., Eds.; CRC Press: Boca Raton, FL, 2007; Chapter 8.
7. Venton, B. J.; Robinson, T. E.; Kennedy, R. T.; Maren, S. Dynamic Amino Acid Increases in the Basolateral Amygdala During Acquisition and Expression of Conditioned Fear. *Eur. J. Neurosci.* **2006**, *23*, 3391–3398.
8. Bayley, H.; Cremer, P. S. Stochastic Sensors Inspired by Biology. *Nature* **2001**, *413*, 226–230.
9. Howorka, S.; Siwy, Z. Nanopore Analytics: Sensing of Single Molecules. *Chem. Soc. Rev.* **2009**, *38*, 2360–2384.
10. Braha, O.; Gu, L.-Q.; Zhou, L.; Lu, X.; Cheley, S.; Bayley, H. Simultaneous Stochastic Sensing of Divalent Metal Ions. *Nat. Biotechnol.* **2000**, *17*, 1005–1007.
11. Cheley, S.; Gu, L.-Q.; Bayley, H. Stochastic Sensing of Nanomolar Inositol 1,4,5-Trisphosphate with an Engineered Pore. *Chem. Biol.* **2002**, *9*, 829–838.
12. Bayley, H.; Jayasinghe, L. Functional Engineered Channels and Pores. *Mol. Membrane Biol.* **2004**, *21*, 209–220.
13. Braha, O.; Walker, B.; Cheley, S.; Kasianowicz, J. J.; Song, L.; Gouaux, J. E.; Bayley, H. Designed Protein Pores as Components for Biosensors. *Chem. Biol.* **1997**, *4*, 497–505.
14. Guan, X.; Gu, L.-Q.; Cheley, S.; Braha, O.; Bayley, H. Stochastic Sensing of TNT with a Genetically Engineered Pore. *ChemBioChem* **2005**, *6*, 1875–1881.
15. Hammerstein, A. F.; Shin, S. H.; Bayley, H. Single-Molecule Kinetics of Two-Step Divalent Cation Chelation. *Angew. Chem., Int. Ed.* **2010**, *49*, 5085–5090.
16. Gu, L.-Q.; Braha, O.; Conlan, S.; Cheley, S.; Bayley, H. Stochastic Sensing of Organic Analytes by a Pore-Forming Protein Containing a Molecular Adapter. *Nature* **1999**, *398*, 686–690.
17. Astier, Y.; Braha, O.; Bayley, H. Toward Single Molecule DNA Sequencing: Direct Identification of Ribonucleoside and Deoxyribonucleoside 5′-Monophosphates by Using an Engineered Protein Nanopore Equipped with a Molecular Adapter. *J. Am. Chem. Soc.* **2006**, *128*, 1705–1710.
18. Shin, S.-H.; Luchian, T.; Cheley, S.; Braha, O.; Bayley, H. Kinetics of a Reversible Covalent Bond-Forming Reaction Observed at the Single-Molecule Level. *Angew. Chem., Int. Ed.* **2002**, *41*, 3707–3709.
19. Wu, H. C.; Bayley, H. Single-Molecule Detection of Nitrogen Mustards by Covalent Reaction within a Protein Nanopore. *J. Am. Chem. Soc.* **2008**, *130*, 6813–6819.
20. Antolini, L.; Marcotrigiano, G.; Menabue, L.; Pellacani, G. C.; Saladini, M.; Sola, M. Coordination Behavior of L-glutamic Acid—Spectroscopic and Structural Properties of (L-Glutamato)(imidazole)copper(II), (L-Glutamato)(2,2′-bipyridine)copper(II), and Aqua(L-glutamato)(1,10-phenanthroline)copper(II) Trihydrate Complexes. *Inorg. Chem.* **1985**, *24*, 3621–3626.
21. Ervin, E. N.; White, R. J.; White, H. S. Sensitivity and Signal Complexity as a Function of the Number of Ion Channels in a Stochastic Sensor. *Anal. Chem.* **2009**, *81*, 533–537.
22. Sanchez-Quesada, J.; Ghadiri, M. R.; Bayley, H.; Braha, O. Cyclic Peptides as Molecular Adapters for a Pore-Forming Protein. *J. Am. Chem. Soc.* **2000**, *122*, 11758–11766.
23. Woodhull, A. M. Ionic Blockage of Sodium Channels in Nerve. *J. Gen. Physiol.* **1973**, *61*, 687–708.
24. Wei, R.; Gatterdam, V.; Wieneke, R.; Tampé, R.; Rant, U. Stochastic Sensing of Proteins with Receptor-Modified Solid-State Nanopores. *Nat. Nanotechnol.* **2012**, *7*, 257–263.
25. Burnstock, G. Cotransmission. *Curr. Opin. Pharmacol.* **2004**, *4*, 47–52.
26. White, R. J.; Ervin, E. N.; Yang, T.; Chen, X.; Daniel, S.; Cremer, P. S.; White, H. S. Single Ion-Channel Recordings Using Glass Nanopore Membranes. *J. Am. Chem. Soc.* **2007**, *129*, 11766–11775.
27. Heitz, B. A.; Xu, J.; Hall, H. K., Jr.; Aspinwall, C. A.; Saavedra, S. S. Enhanced Long-Term Stability for Single Ion Channel Recordings Using Suspended Poly(lipid) Bilayers. *J. Am. Chem. Soc.* **2009**, *131*, 6662–6663.
28. Sawada, K.; Echigo, N.; Juge, N.; Miyaji, T.; Otsuka, M.; Omote, H.; Yamamoto, A.; Moriyama, Y. Identification of a Vesicular Nucleotide Transporter. *Proc. Natl. Acad. Sci. U. S.A.* **2008**, *105*, 5683–5686.
29. Cheley, S.; Malghani, M. S.; Song, L.; Hobaugh, M.; Gouaux, J. E.; Yang, J.; Bayley, H. Spontaneous Oligomerization of a Staphylococcal α-Hemolysin Conformationally Constrained by Removal of Residues that Form the Transmembrane β-Barrel. *Protein Eng.* **1997**, *10*, 1433–1443.