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# High Resolution Physical Characterization of Single Metallic Nanoparticles

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Cover Letter

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Benjamin Werth
Science Editor
JoVE

Dear Mr. Werth,

Attached, please find a manuscript for publication in JoVE entitled: "High Resolution Physical Characterization of Single Metallic Nanoparticles" by Ettedgui, et al. The results should be of interest to experimentalists in electrophysiology, applied science and chemistry communities.

This manuscript reports a new method to detect and characterize of metallic nanoclusters using single molecule nanopore-based measurements. This technique allows the simultaneous determination of multiple species in solution, and is several orders of magnitude more sensitive than conventional analytical techniques. Specifically, we show that a protein nanopore can identify the change in concentration of 12-phosphotungstic acid derivatives induced by pH changes.

The results suggest that nanopore measurements can serve as a complementary approach with enhanced sensitivity to traditional analytical chemistry tools in the study of polyoxometalates and extends analysis to the single molecule limit. This method opens further possibilities in the characterization of metallic clusters.

Thank you for your consideration of this manuscript.

I look forward to hearing from you,

Best Regards,

Jessica Ettedgui, Ph.D.

**TITLE:**

# High Resolution Physical Characterization of Single Metallic Nanoparticles

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**KEYWORDS:**

Nanopore, α-Hemolysin, Lipid bilayer, Single-molecule analysis, Polyoxometalates, Isomers

**SHORT ABSTRACT:**

Here, we present a protocol to detect discrete metal oxygen clusters, polyoxometalates (POMs), at the single molecule limit using a biological nanopore-based electronic platform. The method provides a complementary approach to traditional analytical chemistry tools used in the study of these molecules.

**LONG ABSTRACT:**

Individual molecules can be detected and characterized by measuring the degree by which they reduce the ionic current flowing through a single nanometer-scale pore. The signal is characteristic of the molecule's physicochemical properties and its interactions with the pore. We demonstrate that the nanopore formed by the bacterial protein exotoxin *Staphylococcus aureus* alpha hemolysin (αHL) can detect polyoxometalates (POMs, anionic metal oxygen clusters), at the single molecule limit. Moreover, multiple degradation products of 12-phosphotungstic acid POM (PTA, $H_3PW_{12}O_{40}$) in solution are simultaneously measured. The single molecule sensitivity of the nanopore method allows for POMs to be characterized at significantly lower concentrations than required for nuclear magnetic resonance (NMR) spectroscopy. This technique could serve as a new tool for chemists to study the molecular properties of polyoxometalates or other metallic clusters, to better understand POM synthetic processes, and possibly improve their yield. Hypothetically, the location of a given atom, or the

rotation of a fragment in the molecule, and the metal oxidation state could be investigated with this method. In addition, this new technique has the advantage of allowing the real-time monitoring of molecules in solution. The single molecule sensitivity of the nanopore method allows for POMs to be characterized at significantly lower concentrations than required for NMR spectroscopy and other techniques.

**INTRODUCTION:**

Detecting biomolecular analytes at the single molecule level can be performed by using nanopores and measuring ionic current modulations. Typically, nanopores are divided into two categories based on their fabrication: biological (self-assembled from protein or DNA origami) [1,2,3], or solid-state (*e.g.*, manufactured with semiconductor processing tools)[4,5]. While solid-state nanopores were suggested as potentially more physically robust and offer a wide range of solution conditions, protein nanopores thus far offer greater sensitivity, more resistance to fouling, greater bandwidth, better chemical selectivity, and a greater signal to noise ratio.

A variety of protein ion channels, such as the one formed by *Staphylococcus aureus* α-hemolysin (αHL), can be used to detect single molecules, including ions (*e.g.*, $H^+$ and $D^+$)[2,3], polynucleotides (DNA and RNA)[6-8], damaged DNA[9], polypeptides[10], proteins (folded and unfolded)[11], polymers (polyethylene glycol and others)[12-14], gold nanoparticles[15-19], and other synthetic molecules[20].

We recently demonstrated that the αHL nanopore can also easily detect and characterize metallic clusters, polyoxometalates (POMs), at the single molecule level. POMs are discrete nanoscale anionic metal oxygen clusters that were discovered in 1826[21], and since then, many more have been synthesized. The different sizes, structures, and elemental compositions of polyoxometalates that are now available led to a wide range of properties and applications including chemistry[22,23], catalysis[24], material science[25,26], and biomedical research[27,28 29].

POM synthesis is a self-assembly process typically carried out in water by mixing the stoichiometrically required amounts of monomeric metal salts. Once formed, POMs exhibit a great diversity of sizes and shapes. For example, the Keggin polyanion structure, $XM_{12}O_{40}^{q-}$ is composed of one heteroatom (X) surrounded by four oxygens to form a tetrahedron (q is the charge). The heteroatom is centrally located within a cage formed by 12 octahedral $MO_6$ units (where M = transition metals in their high oxidation state), which are linked to one another by neighboring shared oxygen atoms. While tungsten polyoxometalates structure is stable in acidic conditions, hydroxide ions lead to the hydrolytic cleavage of metal-oxygen (M-O) bonds[30]. This complex process results in the loss of one or more $MO_6$ octahedral subunits, leading to the formation of monovacant and trivacant species and eventually to the complete decomposition of POM. Our discussion here will be limited to the partial decomposition products of 12-phosphotungstic acid at pH 5.5 and 7.5.

The goal of this protocol is to detect discrete metal oxygen clusters at the single molecule limit using a biological nanopore-based electronic platform. The nanopore-based analytical method

allows the detection of metallic clusters in solution. Multiple species in solution can be discriminated with greater sensitivity than conventional analytical methods[33]. With it, subtle differences in POM structure can be elucidated, and at concentrations markedly lower than those required for NMR spectroscopy. Importantly, this approach even allows the discrimination of isomeric forms of $Na_8HPW_9O_{34}$[1].

**PROTOCOL:**

**Note:** The protocol below is specific to the Electronic BioSciences (EBS) Nanopatch DC System. However, it can be readily adapted to other electrophysiology apparatus used to measure the current through planar lipid bilayer membranes (standard lipid bilayer membrane chamber, U-tube geometry, pulled microcapillaries, *etc.*). The identification of commercial materials and their sources is given to describe the experimental results. In no case does this identification imply recommendation by the National Institute of Standards and Technology, nor does it imply that the materials are the best available.

**1. Solution and Analyte Preparation**

1.1. Prepare all electrolyte solutions with 18 MΩ-cm water from a Type-1 water purification system to remove trace organic species and then filter all electrolyte solutions through a 0.22 µm vacuum filter immediately before ion channel recordings.

**Note:** The water quality is a critical factor for the stability and longevity of the membrane nanopore system.

1.2. Wild Type αHL.

1.2.1. Follow MSDS precautions when handling the αHL toxin protein.

1.2.2. Mix lyophilized wild-type monomeric *S. aureus α*-Hemolysin (*α*HL) powder with 18 MΩ-cm water at 1 mg/mL. Distribute 10 to 30 µL aliquots of the sample into cryo-safe centrifuge tubes, quickly flash freeze in liquid nitrogen and then store at -80 °C. Alternatively, use purified preformed heptameric *α*HL[31].

1.3. Dissolve the lipid 1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine (DPhyPC) to 0.2 mg/mL in *n*-decane in a 4 mL glass scintillation vial with a polytetrafluoroethyleneeflon-coated cap. Store the solution at 4 °C for repeated use for up to one month.

1.4. Prepare phosphotungstic acid solutions.

1.4.1. Prepare a 2 mM phosphotungstic acid stock solution by dissolving 57.6 mg of $H_3PW_{12}O_{40}$ into 10 mL of a 1 M NaCl and 10 mM $NaH_2PO_4$ solution, which constitutes the stock solution.

1.4.2. Take 5 mL of this solution and adjust the pH to 5.5 with 3 M NaOH. Adjust the pH of the other 5 mL of the stock solution to 7.5 with 3 M NaOH.

**Note:** At pH 5.5, 12-phosphotungstic acid (PTA, $H_3PW_{12}O_{40}$) decomposes primarily into the monovacant anion $[PW_{11}O_{39}]^{7-}$.

**2. Test Cell Assembly**

2.1. Assemble the test cell per the manufacturer's instructions.

2.2. Soak one Ag/AgCl wire in bleach (sodium hypochlorite) for 10 minutes after abrading it with 600 grit sandpaper. Position the electrode inside the quartz nanopore membrane (QNM).

2.3. Place a cylindrical AgCl pellet electrode embedded in a silver wire outside of the QNM.

2.4. Once the test cell is set up, turn on the power supply and data acquisition program. Ensure that the DC current reading is 0 pA in the absence of solution in the test cell.

2.5. Use a syringe connected to the test cell *via* a fluid line to add buffered electrolyte solution above the face of the QNM and to ensure that the ionic current saturates the amplifier. If it does not, the QNM may be clogged. Apply a pop voltage (± 1 V) and/or a pressure greater than 300 mm Hg to clear it. If that works, reduce the voltage and pressure.

3. **Lipid Bilayer Formation**

3.1. Fill the solution in the test cell so that the solution level is well above the face of the QNM. Then lower the solution level *via* syringe to below the face, such that the current decreases to zero.

3.2. Dip a 10 µL pipette tip into the lipid vial. Push on the back end of the pipette tip and tap it on the side of the vial to remove all visible lipid.

3.3. Touch the pipette tip onto the air-water interface of the solution in the test cell when the solution level is above the QNM's face and wait two to five minutes for the lipid to spread uniformly.

3.4. Slowly lower the solution level below the face of the QNM until the current saturates, and then slowly raise the solution level past the face of the QNM to form a lipid bilayer membrane.

3.4.1. Once a bilayer appears to form (*i.e.,* when the current goes to zero), try popping it several times by increasing the pressure and ensure that the QNM is not clogged. To reform the lipid bilayer membrane, lower again the solution level below the face of the QNM and slowly raise it.

3.5. If the lipid bilayer membrane did not form the first time, lower the solution below the face and raise it again. If it does not form after 3 trials, add some lipids again as described in 3.2 and 3.3.

3.6. After forming a membrane, set the current offset to zero when the applied potential is zero.

4. ***α*HL Pore Formation**

4.1. Add 2.5 ng of purified preformed *α*HL heptameric protein sample to the test cell (volume ≈ 200 µL) to enable insertion of the protein or alternatively 250 ng of monomeric αHL.

4.2. Increase the pressure on the bilayer with a gas-tight syringe (**Figure 1**) after a bilayer is formed, to expand the membrane from the QNM, and facilitate nanopore insertion. Raise the applied back pressure typically between 40 to 200 mmHg, depending on each QNM.

**Note:** The EBS software has an automated insertion feature that applies a higher bias (typically 200 to 400 mV) to induce pore formation and then automatically reduces the desired voltage to the measurement bias once a single channel forms.

4.3. After a nanopore forms, reduce the back-pressure to about 1⁄2 of the insertion pressure. If multiple channels are observed, remove them by significantly reducing the pressure.

5. **Metallic Cluster Partitioning in the Nanopore**

5.1. To account for electrode imbalances, set the DC offset voltage such that when the applied potential is set to zero there is no measured current.

5.2. Prior to adding the POM sample, perform a control experiment to ensure there are no contaminants (*e.g.*, trace POMs from a previous experiment) in the reservoir. Specifically, acquire an ionic current trace under an applied potential of +120 mV to -120 mV in the absence of any POMs to verify that no spontaneous current blockades are present.

**Note:** Due to the asymmetric structure of the αHL channel (**Figure 1**), above a critical voltage rectification will be observed and the measured ionic current flowing the channel will differ for positive and negative applied potentials. The ratio of the measured current above this applied voltage is indicative of the orientation of the *α*HL nanopore in the membrane.

5.3. Add the POM sample by flushing the reservoir with metallic cluster solution at 1 to 5 µM concentration. Alternatively, load the sample into the capillary prior to cell assembly to study the partitioning of POMs into the other end of the αHL channel.

5.4. Record the ionic current using the manufacturer's software to detect transient current blockades caused by partitioning of individual POMs into the nanopore. Estimate the physical and chemical properties of the molecule from the ionic current blockade depth, event frequency, and the residence time distribution of the blockades.

**6. Ion Channel Recordings and Data Analysis**

6.1. Acquire the ionic current time series measurements using a high-impedance, low-noise amplifier and data acquisition system. Perform the measurements at an applied voltage of -120 mV (relative to channel *cis* side) for each pH.

6.2. Apply a low-pas 100 kHz 8-pole Bessel filter to the signal, which is subsequently digitized at 500 kHz (*i.e.,* 2 μs/point). Extract events from the time series and analyze events using the ADEPT algorithm in the *MOSAIC* software package[32,33].

**REPRESENTATIVE RESULTS:**

Over the past two decades, membrane-bound protein nanometer-scale pores have been demonstrated as versatile single-molecule sensors. Such nanopore measurements are conceptually simple: two chambers filled with electrolyte solution are separated by a nanopore embedded in an electrically insulating lipid membrane. Either a patch-clamp amplifier or an external power supply provides an electrostatic potential across the nanopore *via* Ag/AgCl electrodes immersed in the electrolyte reservoirs. The electric field drives individual charged particles into the pore, which produces transient reductions in the ionic current that depend on the size, shape, and charge of the particles. A computer program controls the applied voltage and monitors, in real time, the ionic current blockades caused by molecules reversibly partitioning into the pore. The current is amplified and converted to voltage with a low-noise, high impedance field-effect transistor and digitized using a data acquisition card.

Here, we provide a general procedure for detecting polyoxometalates with a biological nanopore. As seen in **Figure 2**, prior to the addition of POMs the unobstructed channel has a mean open channel current of ~ 100 pA at an applied potential of -120 mV. The addition of POMs produces transient blockades and decreases the ionic current by approximately 80%. As expected, because these particles are negatively charged, the blockades are not observed when the polarity of the applied potential is reversed. Note that if the POMs didn't interact with the pore wall, they would diffuse through the pore's in about 100 ns, which is far too brief to be detected with a conventional patch clamp amplifier. Thus, most of the time a given particle spends in the pore is a direct consequence of the interaction between the particle and the pore. The duration of an ionic current blockade event is defined as the residence time, tau ($\tau$).

To illustrate the utility of this method, we discuss the possibility for an αHL nanopore to monitor the decomposition of 12-phosphotungstic acid (PTA, $H_3PW_{12}O_{40}$) at pH 5.5 and pH 7.5. This decomposition can be observed with $^{31}P$ NMR measurements, but the concentration

needed is 2 mM while nanopore measurements need only 30 μM, because of the sensitivity of the nanopore measurement. At pH 5.5, $[PW_{11}O_{39}]^{7-}$ is the predominant species[30].

The data analysis is performed by calculating a histogram of the relative blockade depth ratio (*i.e.,* $\langle i \rangle / \langle i_o \rangle$, where $\langle i \rangle$ is the mean current with the POM in the pore and $\langle i_o \rangle$ is the mean open channel current). The histogram of the mean current blockade depth ratios at -120 mV and pH 5.5 exhibits a minor peak at $\langle i \rangle / \langle i_o \rangle \approx 0.06$ and major peak at $\langle i \rangle / \langle i_o \rangle \approx 0.16$ (**Figure 3**, *green*). We assume these peaks correspond to $[P_2W_5O_{23}]^{6-}$ and $[PW_{11}O_{39}]^{7-}$, respectively, based on $^{31}P$ NMR. $^{31}P$ NMR studies suggest that increasing the pH changes the relative concentration of these two species, and this is borne out by the change in the area of the two peaks shown in **Figure 3**.

When the POM solution is titrated to pH 7.5 *ex situ*, the total POM concentration decreases due to the partial degradation of the two-principal species to inorganic salts (*i.e.,* free phosphate, $H_xPO_4^{-3+x}$ and tungstate, $WO_4^{2-}$ ions). The histogram of the relative blockade depth ratio also shows two principal peaks (**Figure 3**, *orange*), but with 20-fold fewer events (which suggests the total POM concentration at pH 7.5 is approximately 20-fold less than that at pH 5.5, if the nanopore's capture efficiency for POMs is the same at the two pH values). It is interesting to note that at pH 7.5 and greater, the POM species observed here were not detected in the $^{31}P$ NMR spectrum due to their low concentration caused by their dissociation into phosphate and tungstate ions.

Each event's residence time in the pore is defined by the duration of the individual ionic current blockades. The distribution of residence times provides insight into the different species that are present. It was shown earlier that for blockades caused by a differently-sized polymers of poly(ethylene glycol), the residence time distribution for each size of that polymer is well described by a single exponential. That result suggests the interaction of that polymer is a simple reversible chemical reaction[12, 13, 20].

**Figure 4** illustrates that the residence time distributions for the two peaks were well differentiated at pH 5.5 and 7.5. Two features are clear. First, under all conditions, multiple exponentials are required to fit each of the distributions, which suggests there are variations of the POMs within each species. Second, the residence times of the POMs in the pore are much shorter at pH 7.5 compared to those at pH 5.5, which suggests a weakening of the interaction between the pore and POMs. It has been shown previously that a change in pH alters the relative number of fixed charges in or near the $\alpha$HL channel lumen. These changes will directly alter the interactions with partitioning POMs inside the pore and therefore modify their residence times[34,35].

**FIGURE LEGENDS:**

**Figure 1: Schematic diagram of the experimental setup.** Method for nanopore-based characterization of individual polyoxometalate molecules. A protein nanopore that self-assembles in a 4 nm thick lipid bilayer membrane is bathed by aqueous electrolyte solutions in a glass capillary and larger reservoir. A pressure is applied to the glass capillary with a gas tight

syringe to aid nanopore incorporation. A potential *V* is applied across the membrane with a matched pair of Ag/AgCl electrodes and drives an ionic current (*e.g.*, $Na^+$ and $Cl^-$) through the pore. The current is converted to voltage with a high impedance amplifier, digitized with an analog to digital converter (ADC) and stored on a computer. Computer software controls the applied potential through a digital to analog converter (DAC) and monitors, in real time, the transient current blockades caused by single molecules that partition into the pore.

**Figure 2: Nanopore-based detection of individual metallo-nanoparticles.** An illustration of ionic current time series traces that occur before and after the addition of a POM solution to the nanopore apparatus. The partitioning of individual anionic POMs into the pore causes transient current reductions in the mean open pore current, $\langle i_o \rangle$. (*Right*) A typical event, illustrating the mean current of the blockade ($\langle i \rangle$) and the residence time ($\tau$) of the particle in the pore. The applied potential was -120 mV, and the solutions contained 1 M NaCl, 10 mM $NaH_2PO_4$ at pH 5.5. The *cis* compartment also contained 30 µM of 12-phosphotungstic acid. The current blockade depth ratio ($\langle i \rangle / \langle i_o \rangle$) and the residence times ($\tau$) provide information about which POM species are present in solution. Under the conditions we used here, the αHL channel does not gate (spontaneously close) when POMs are not present.

**Figure 3: Histograms of the current blockade depth ratio at pH 5.5 and 7.5**. Histograms of the POM-induced ionic current blockade depth ratio at pH 5.5 (*green*) and 7.5 (*orange*) with an applied potential *V* = -120 mV. The two peaks present at each pH value correspond to the known predominate POM species in solution under those conditions. The current blockade depth ratios of 0 and 1 correspond to a fully blocked and open pore, respectively. The histograms were created with a bin width of 0.001 and normalized to counts/s by dividing by the data acquisition time.

**Figure 4: Residence time distribution and fitting with several exponentials.** The distribution of residence times for POM-induced current blockades caused by the two-principal species (peaks 1 and 2 in **Figure 4**) observed at pH 5.5 and 7.5 in a semi-log plot. For both species, the residence times are markedly shorter at the higher pH value, which suggests the interaction between the pore and POMs changed. The solid lines are fits of an exponential mixture model to the data.

**DISCUSSION:**

Due to their anionic charge, POMs likely associate with organic counter cations through electrostatic interactions. Therefore, it is important to identify the proper solution conditions and the right electrolyte environments (especially cations in solution) to avoid complex formation with POMs. Particular care is required in the buffer choice. For example, the capture rate of POMs with tris(hydroxymethyl)aminomethane and citric acid-buffered solutions is significantly lower than that in phosphate buffered solution, likely because the first two buffers form a complex with the POM that doesn't strongly interact with the nanopore. Moreover, the NaCl electrolyte was purposely used instead of KCl (as well as the other alkali metals) to avoid the precipitation of $[PW_{11}O_{39}]^{7-}$ by $K^+$.

Critical to the accurate measurement of the residence time distributions is the ability to measure the current at a sufficiently high bandwidth. For instance, with exponentially-distributed residence times there are far more blockades with short than long residence times, and an accurate estimation of the residence time distributions is better achieved by collecting a great deal of data (*i.e.,* acquiring it at as high as a bandwidth the system's electrical capacitance allows). To achieve this condition in nanopore spectroscopy, the system capacitance (membrane and stray capacitance) should be minimized. Stray capacitance is reduced by decreasing the length of all connecting cables and using high quality electrical contacts. The membrane capacitance is minimized by decreasing the surface area of the bilayer membrane, increasing the thickness of supporting materials (*i.e.,* quartz, polytetrafluoroethylene, *etc.*), and decreasing the area of exposed supporting materials to the electrolyte. In practice, a typical instrument's stray capacitance (≈ 2 pF) will limit the noise for membranes < 1 to 5 μm in diameter. This constitutes the method's limitation. For example, the detection of small and highly charged single molecules can be challenging due to their relatively short residence time.

The mechanism by which pressure enables control of channel insertion is not completely understood. The quartz microcapillaries have a very small diameter on which the membrane is formed. Applying pressure will cause the membrane to bulge (thereby increasing the membrane surface area) and possibly thin the membrane. Both effects would increase the rate at which channels will form in the membrane. When a single channel spontaneously forms, reduce the pressure to prevent the insertion of additional channels. The removal of non-inserted HL from the bulk aqueous phase is not required if the $\alpha$HL concentration is sufficiently low.

The structures and charges of polyoxometalates are currently studied using traditional analytical chemistry techniques, including NMR, Ultraviolet–visible, Infrared and Raman spectroscopy, mass spectrometry, and X-ray diffraction. We expect that nanopore measurements will complement the characterization of these and other physical properties of POMs, as well as the study of their speciation at low concentration, which will help better understand the synthetic pathway of polyoxometalates formation. It was also shown previously that the $\alpha$HL pore can even distinguish between 2 isomers of the trivacant Keggin form $Na_8HPW_9O_{34}$[30].

In summary, we have shown that a membrane-bound protein nanopore can be used to detect and characterize tungsten oxide metallic clusters (heteropolytungstates) in solution using a simple high-resolution electrical measurement. The sensitivity afforded by this novel approach permits the tracking of subtle differences in POM structure that arise at different pH values at concentrations that are substantially lower (> 70-fold) than required for traditional methods such as NMR spectroscopy. Due to the single molecule detection capability of nanopores, the actual limit of detection in the method can be made much lower by measuring the current for longer times (the capture rate scales in proportion to the POM concentration).

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**DISCLOSURES:**

None.

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Figure 1

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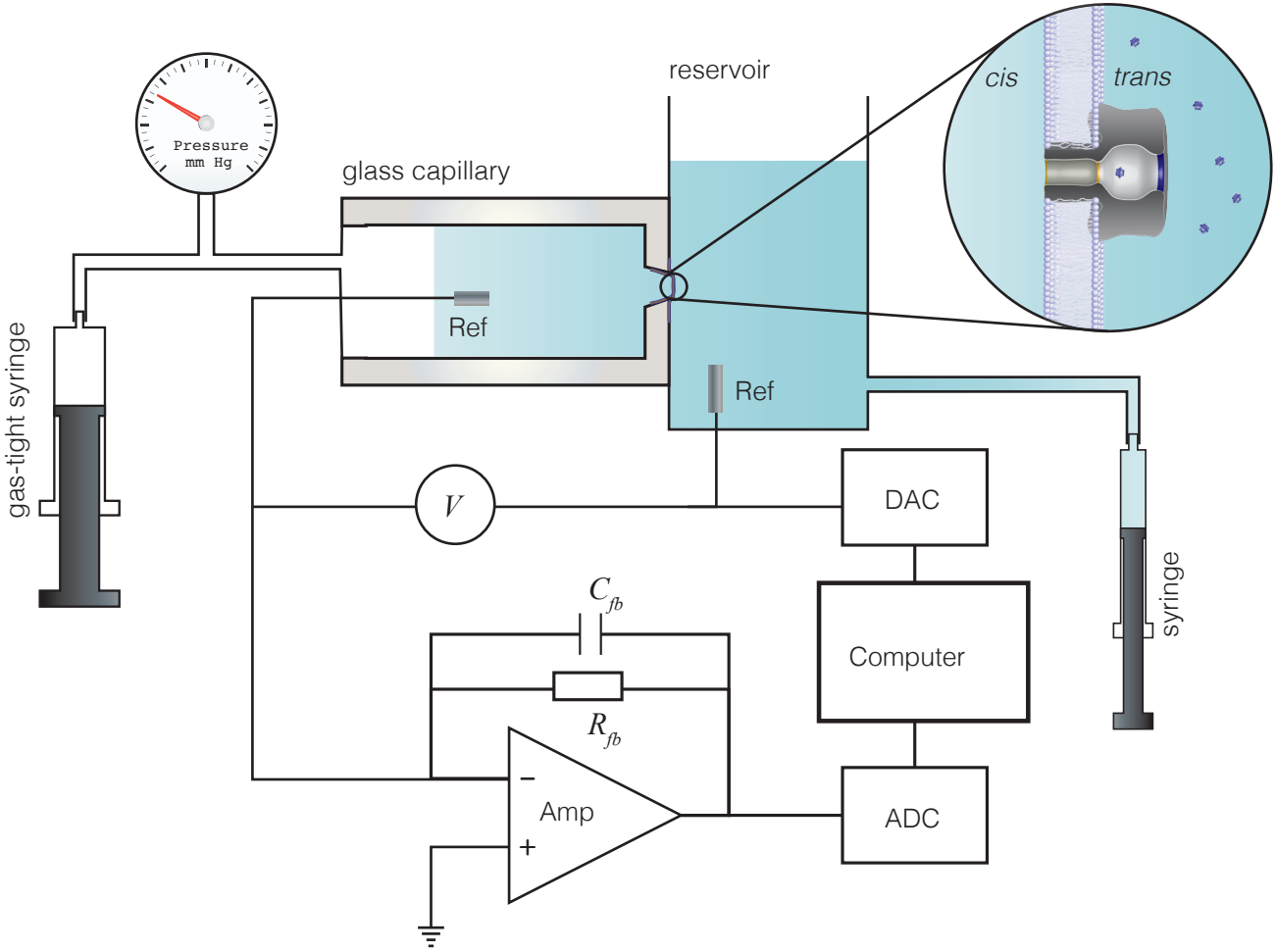

Figure 2

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Figure 3

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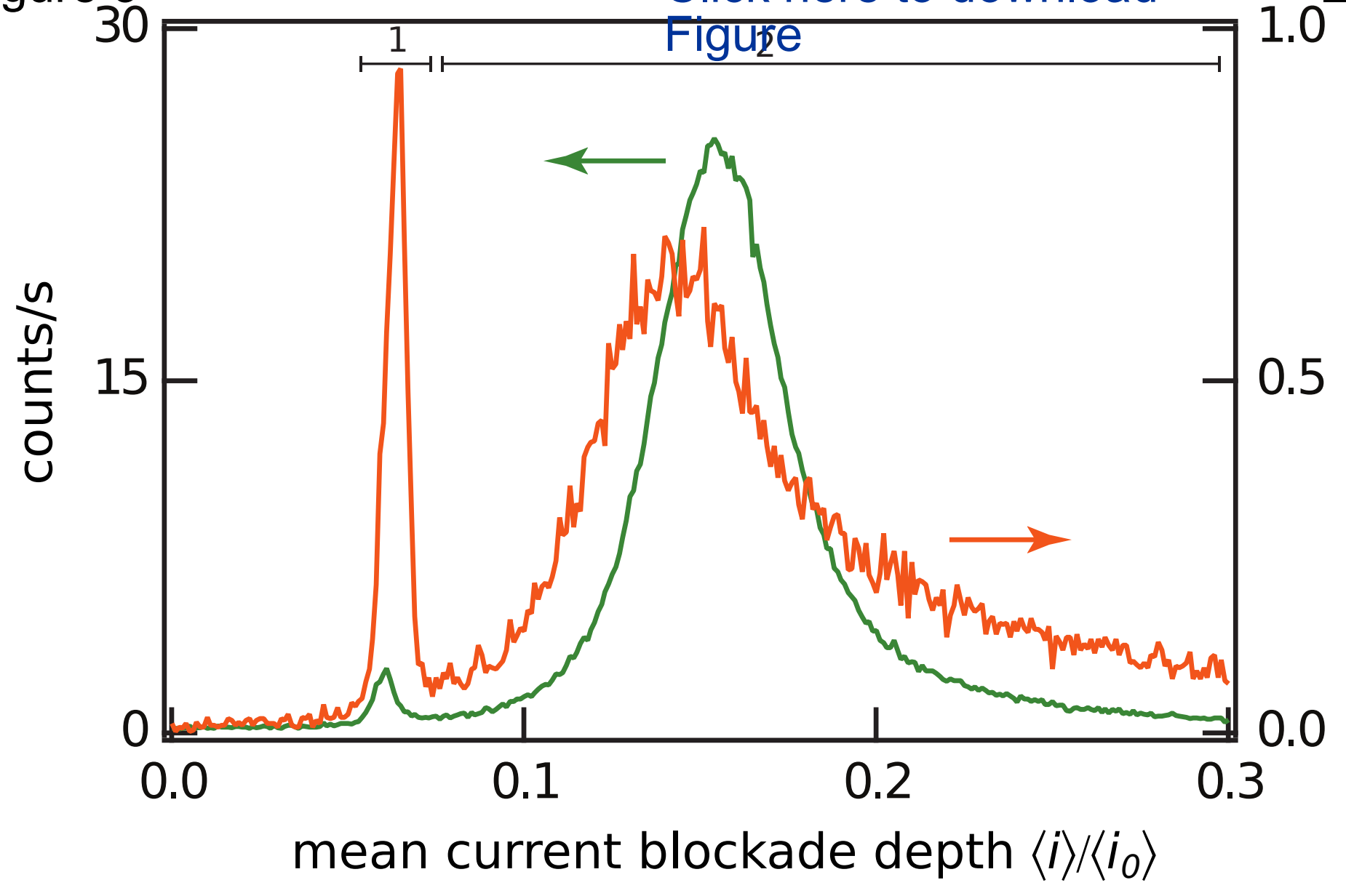
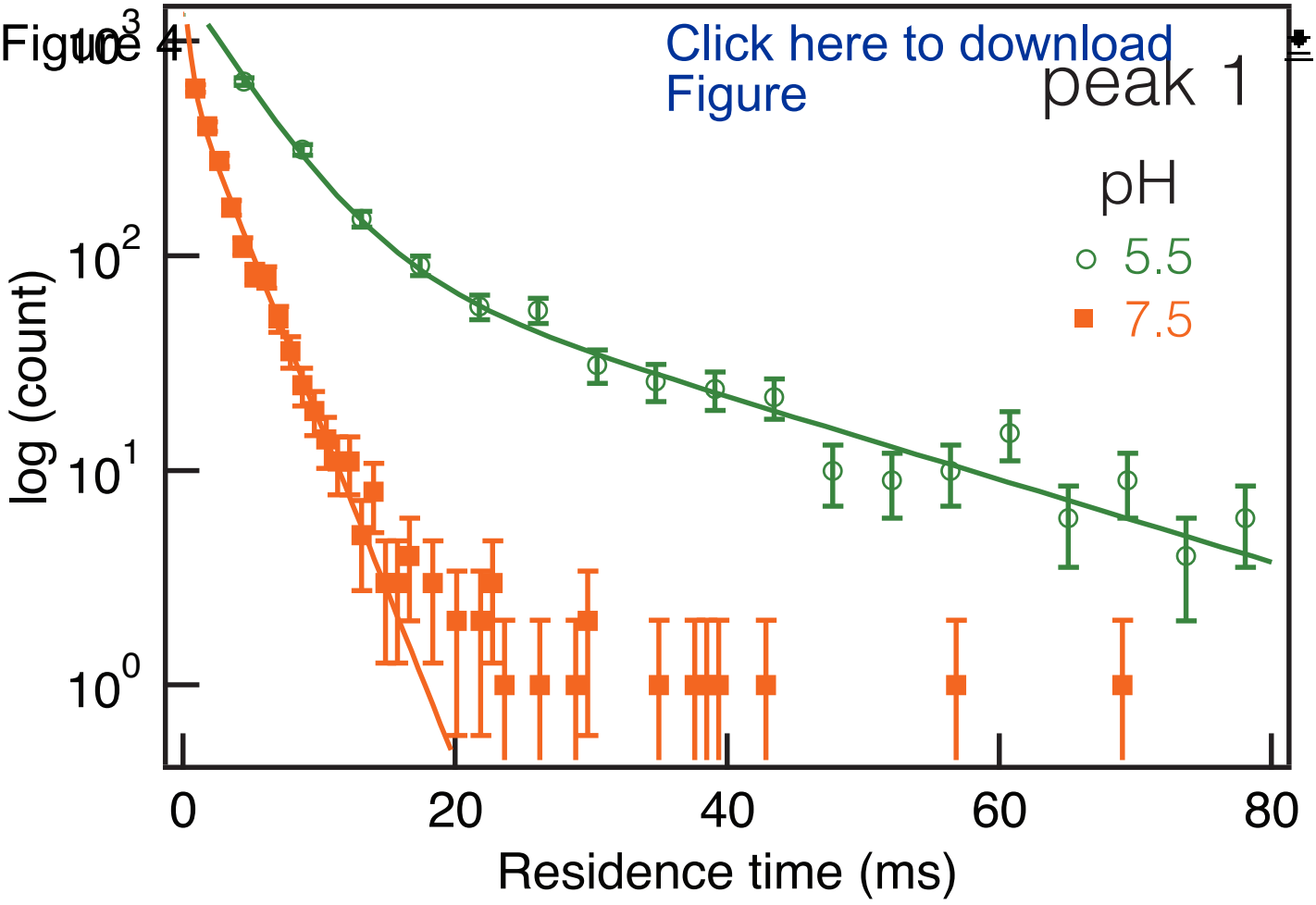
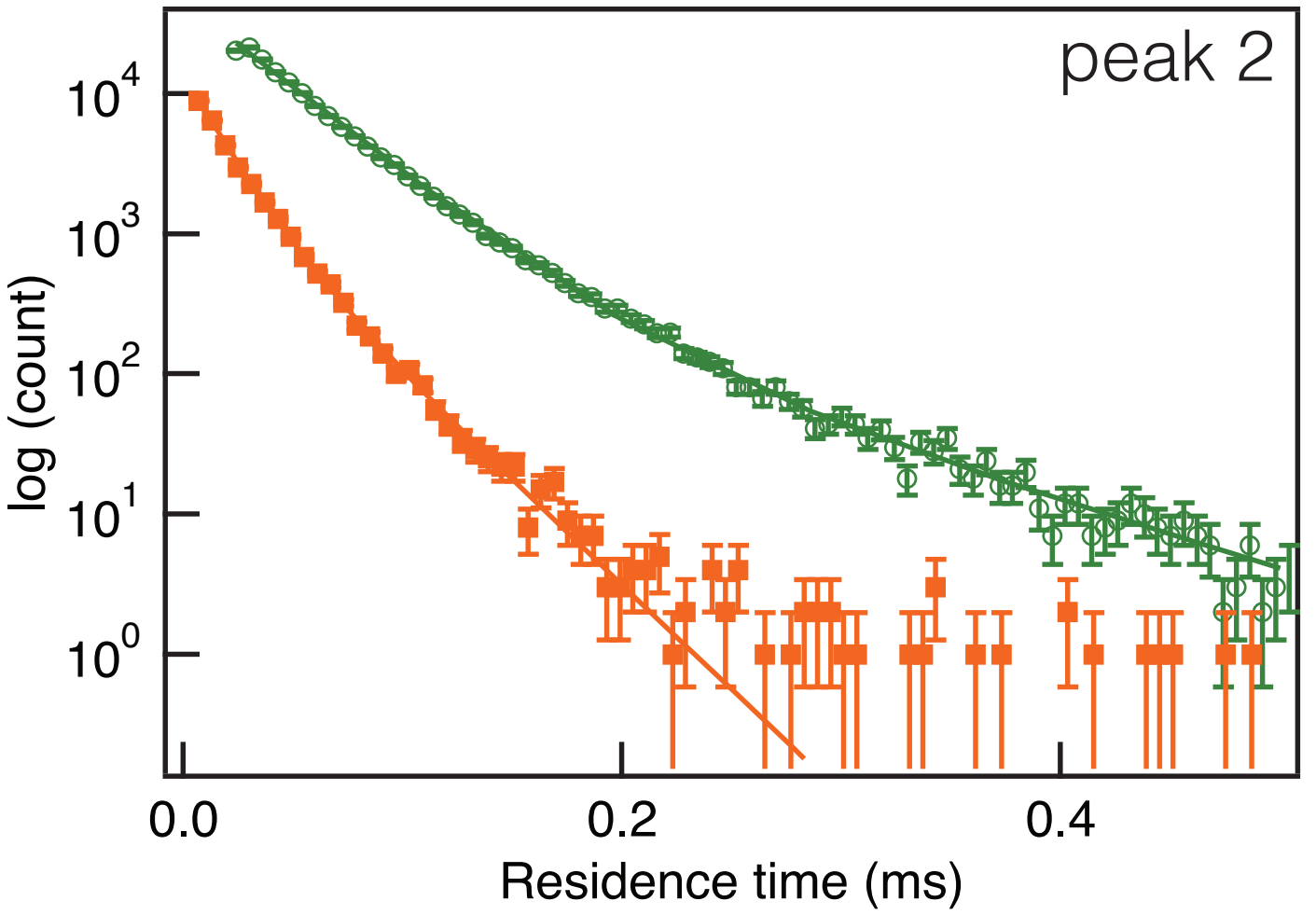


Figure 4





| Name of Material/ Equipment | Company |
|---|---|
| Nanopatch DC System | Electronic Biosciences, Inc., EBS |
| Millipore LC-PAK | Millipore vacuum filter |
| 1,2-Diphytanoyl-sn- Glycero-3-Phosphocholine (DPhPC) | Avanti Polar Lipids, Alabaster, AL |
| *Decane, ReagentPlus, ≥99%,* | Sigma-Aldrich |
| αHL | List Biological Laboratories, Campbell, CA |
| Ag wire | Alfa Aesar |
| 2 mm Ag/AgCl disk electrode | In Vivo Metric |
| High-impedance amplifier system | Electronic Biosciences, San Diego, CA |
| quartz capillaries | |
| custom polycarbonate test cell | |
| Data Processing and Analysis MOSAIC | https://pages.nist.gov/mosaic/ |
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| Phosphotungstic acid hydrate | Sigma-Aldrich |
| Sodium Chloride | Sigma-Aldrich |
| sodium phosphate monobasic monohydrate | Sigma-Aldrich |

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| 850356P | |
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**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Figure 4: Please use the same y scale for peak 1 and peak 2 if possible. Please use the same notation for numbers (1000 or 103, etc.) in the figure.
We made the necessary changes to figure 4.

3. Please include a space between all numbers and their corresponding units: 10 mM, 120 mV, etc.
We made the necessary changes.

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”
We changed the text to clarify this point.

5. Introduction: Please include a clear statement of the overall goal of this method.
We revised the introduction to improve the clarity of the overall goal.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: NanopatchTM 106 DC System, Millipore LC-PAK, Millipore vacuum filter, List labs, Inc., Avanti Polar Lipids, Sigma-Aldrich, etc.
We made the necessary changes.

7. 3.2: Please break up into substeps if possible.
We made the necessary changes.

8. Discussion: Please describe any limitations of the technique.
We revised the discussion and clarify this point.

9. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
We made the necessary changes.

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:
The manuscript describes a method to measure various species of polyoxometalates (POM) present in solution by means of a nano-pore detector inserted in a lipid bilayer membrane. The use of a single nano-pore enables detection of individual POMs as they transiently block the electric current through the pore during their electrolytic passage. The extend of the blockage reports on the type of chemical species, i.e. particle size, and the frequency on the concentration of the respective POM variant.
The presented method is advantageous to other bulk methods as it quantitatively resolves population distributions of various species simultaneously present in solution. The protocol and setup is straight forward and can be used to analyze various kinds of chemical compounds that form nano-clusters.
The manuscript is very well written and easily understandable even for non-chemists.

Major Concerns:
Line 306/Figure2: Only one trace is shown, the one after addition of POM solution. As mentioned in the figure legend, a trace of before addition of POMs would be nice for comparison. That would illustrate if there are any spontaneous pore closure of HL occurring or are all current changes due to the POMs?
Under the conditions we used here, the alpha HL channel does not gate (spontaneously close) when POMs are not present. We revised Figure 2 and the text to address this question.

Minor Concerns:

171-172: Is the popping of the membrane reversible? Can you break the membrane and it re-seals automatically? Please clarify.
We clarified that point in the text.

184-185: Why is this pressure needed of HL insertion? HL inserts spontaneously into all kind of biological lipid membranes. Why is this not the case for DPHyPC. Some reader may not know why you use this specific lipid (proton tight etc.). Please explain.

The mechanism by which pressure enables control of channel insertion is not completely understood. The quartz microcapillaries have a very small diameter on which the membrane is formed. Applying pressure undoubtedly bulges the membrane outward, which may help thin the membrane and certainly increases its surface area. Both effects would increase the rate at which channels will form in the membrane. When a single channel spontaneously forms, we reduce the pressure to prevent the insertion of additional channels.

191-192: HL pops out from the membrane if back pressure is reduced?
If the back pressure is reduced too much, all of the pores will be removed.

191-193: Removal of non-inserted, soluble HL from the reservoir is not required because it doesn't insert without increased back pressure?

Yes, the removal of non-inserted HL from the bulk aqueous phase is not required **if** the protein concentration is sufficiently low.
We addressed this comment in the text.

212: What kind of software? Does it come with the device or from the manufacturer?
The manufacturer provides the data acquisition software, which allows to control the device. The data analysis software was written by our lab.

218: Since this is the first time this company is mentioned please spell it out. I was not aware it will be mentioned in the apendix and tried to google it and did not find it this way.
It is our understanding that JoVE cannot publish manuscripts containing commercial language, including trademark symbols (™), registered symbols (®), and company names.

249: insert (tau) to ease transition to figure
done

252: delete "how". Confusing sentence structure.
done

254: Clarify that no NMR data are shown in Fig. 3. It reads as if the NMR is part of the method. Otherwise confusing.
Agreed and altered the text.

259: I find the term 'blockade depth ratio' confusing. Depth is already ratio (i/io). 'Depth ratio' means a ratio of depths to me.
Our lab pioneered the nanopore-based analytical method over 20 years ago and has used the term “blockade depth ratio” since then. However, we changed the text to “relative blockade depth ratio”.

261/Figure 3: Label the x-axis "mean current blockade depth (i/io)"
We changed the x-axis label of figure 3.

318: Which peak corresponds to which species? Smaller molecules block less/shorter? Why is one peak narrow, the other wide?
We do not completely understand what controls the width of the POM-induced current blockade depth histogram. We discussed that point in a recent review and are currently trying to address it experimentally and theoretically.

Our lab previously demonstrated that larger polymers reduce the pore conductance more (and reside longer in the pore) than do smaller ones (PNAS 2007, 2010) and that the analyte-induced pore conductance decrease can be due to volume exclusion and the binding of mobile ions to the molecule in the pore. We assume the same is true here.

320: The concentration of the two POM species is relative to the area of the peaks, not the height.
The POM species concentrations are proportional to the area under each peak in the blockade depth histogram and the peak height. We clarified this in the text.

**Reviewer #2:**
Manuscript Summary:
In this paper, the authors present a protocol for measuring nanoparticles in solution using alpha-hemolysin nanopore sensors. This a useful protocol and an interesting application. At a high level, I would note that the protocol is primarily about setting up the bilayer and hemolysin experiment. The elements of the protocol which are specific to metallic nanoparticles are extremely brief.
We have elaborated further the protocol for the preparation of the metallic nanoparticles.

Major Concerns:
1. The abstract says "Conceivably, the location of a given atom, or the rotation of a fragment in the molecule, and the metal oxidation state could be investigated with this method." Is this an affirmative statement, or a hypothetical?
This is a hypothetical statement and we have clarified this in the text.

2. What advantage does "real-time" analysis have for nanoparticles?
Conceivably, a real-time analysis would allow a direct measurement of the *kinetic and thermodynamic (i.e., steady-state)* changes to the nanoparticles caused by altering the pH, etc. Specifically, it could help identify intermediate states or species, which cannot be observed in an steady-state. It could also provide information on the reaction kinetics, which is invaluable. Ultimately, the real-time analysis could bring crucial information on rearrangements of atoms in those clusters while modifying the pH.

3. Preformed heptameric aHL and monomeric aHL are both described, but which one is demonstrated here? The materials and preparations seem like monomeric, but in the protocol it seems like you use heptameric.
We used both and the only noticeable difference was the concentration required to obtain a single channel in the membrane. When using heptameric HL the concentration is hundred time lower than the monomeric HL. Probably not all monomeric HL assemble in a well-organized homo-heptameric nanopore that can insert correctly in a lipid bilayer membrane.

4. You write that the blockades "provide information about the POM's physical and chemical properties." Can you be more specific? How should the data be analyzed?
Blockades can be analyzed by considering their residence time, which is the time a molecule spend in the pore and their depth, how much a molecule block the pore. These two parameters are dependent on the physical properties and chemical properties of a molecule.

We demonstrated experimentally and theoretically that the blockade depth and mean residence time depend on the physical properties and chemical properties of the molecule in the pore. Specifically, we showed that polymers of poly(ethylene glycol) reduce the aHL channel current due to volume exclusion and the binding of cations to the polymers (PNAS 2007, 2010). Those studies also showed that the mean residence time of PEGs in the pore was a function of the applied potential, the polymer size, and the binding of cations to the polymer.

Because the POMs we used here cause current blockades with two major mean depths and distinctly different residence time distributions, we assume those differences are due to the different physical and chemical properties of the nanoparticles.

In our 2016 JACS paper, we showed that with the blockade depth and residence time distribution data, the aHL nanopore can (surprisingly) discriminate between two different POMs isomers. At this time, we do not completely understand why the pore can separate these two species so well.

Minor Concerns:
5. Please make it clearer earlier on which other papers (only reference 30?) use the described protocol.
We cited this work earlier in the paper

6. A software filter can only be after the digitizer, not before. Please clarify this description.
The manufacturer's data acquisition software applies filters to the data when it reconstructs the current time series.

7. In Fig 1, please clarify the V label. This might be misunderstood as a voltage source, where it is a measurement.
The *V* label is indeed an applied voltage between the two chambers. a patch-clamp amplifier or an external power supply applies an electrostatic potential across the nanopore via Ag/AgCl electrodes immersed in the electrolyte reservoirs.

**Reviewer #3:**
Manuscript Summary:
The manuscript by Jessica Ettedgui and colleagues entitled « High Resolution Physical Characterization of Single Metallic Nanoparticles » describes a very interesting performed study and a very detailled protocol to study the molecular properties of metallic clusters at single molecule level through a solitary nanopore with high resolution. The authors demonstrate that aHL nanopore enables detection of metallic clusters as well as the degradation products of the metallic clusters at different charge states (pH 5.5 and 7.5). Authors compare their results obtained with nanopore measurement to conventional NMR measurements. At pH 5.5 two degradation products were detected with nanopore measurements and identified based on NMR measurements. At pH 7.5 and greater well defined degradation products were detected with nanopore measurements, while NMR measurements failed to detect these degradation

products, mainly because of low abondance of the degradation products. Authors demonstrate clearly that the single molecule sensitivity of nanopore method enables detection of molecules at lower concentration required for NMR spectrometry. To the best of my knowledge, such analysis has not been undertaken before. The study report new results and helpful tips that will be of interest to the wide audience. The presentation is clear and the language is fluent and precise. I think the manuscript will be an excellent contribution to the literature.
I recommend this paper for publication.

Major Concerns:
Nothing

Minor Concerns: In the context of nanopore sensitivity to detect low abundance elements, it was demonstrated more recently that nanopore measurement allows detection and characterization of a few dozens of peptide impurities in a high purity commercial peptide sample, while conventional analysis techniques fail to do so. (doi:10.1038/s41467-018-03418-2). Please cite this work in that context.
We added this reference.

**Reviewer #4:**
Manuscript Summary:
The authors mentioned that this is a new tool and it looked like it is a paper which introduced a new method for PMO detection at a single molecule level. But they published a similar work back in 2016. With the same pore (α-HL) and PMO (Tungsten oxide metallic clusters) and with more data back then. What is the difference between their current work and their work reported at JACS in 2016 https://pubs.acs.org/doi/pdfplus/10.1021/jacs.6b02917
The purpose of publishing a paper in JoVE is to describe experimental methods in a video format. The detection of POMs (polyoxometalates) in solution using a biological nanopore was never described before our work reported in 2016. These experiments involve an unfamiliar technique and *JoVE* videos are a step-by-step visual guide of the actual experiment so that the minute hand movements and other subtle manipulations necessary to perform the experiment successfully can be seen.

The data presented is not enough to support their claim unless they have a supplementary data. Most importantly in their results section, there is no information how many experiments they have done for each data they present (no statistical info!).
This manuscript explains how to get measurements done with a HL nanopore. Its purpose is mainly tutorial. In our JACSs 2016 paper, consequently rich data is accessible as well as statistics. (about 600 000 events measured).

In figure 4, they show the peaks at different figures which is fine to show detail differences, but at least their scaling should be the same (the range of y (log (count)) has to be the same, maximum 10000). In addition, they have to use the same notation (one is exponential and the

other decimal). The x range is ok since the residence time of peak 2 is very small and it can't be visible if they use the same x-range.
We agreed and fixed it.

Their last sentence before acknowledgement is not clear, do they meant "the actual limit of detection can be increased by measuring the current for longer times?"
Due to the single molecule detection nature of nanopore recordings, the actual limit of detection in that method is potentially much lower. As long as the transient current blockades are solely caused by POMs, we can detect POMs at far lower concentrations than shown in the manuscript by measuring the current for longer times (the capture rate scales in proportion to the POM concentration).