**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).