Faraday Discussions
Cite this: *Faraday Discuss.*, 2016, **193**, 141

From single cells to single molecules: general discussion

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DOI: 10.1039/c6fd90066f

Mario A. Alpuche-Aviles opened a general discussion of the papers by Serge Lemay: The concentrations have to be very low and one worries about background issues and the effect of contaminants. Why do they have to be this low? Could these experiments be performed at higher concentrations?

Serge Lemay replied: We would love to be able to work at higher concentrations! The concentration is currently set by the need to work under conditions where there is less than one molecule on average present inside the device. This is needed because the finite time resolution of femtoampere-level electronics leads to blurring of single-molecule signals if they overlap significantly in time.[1] The device volume, in particular the channel width and height, are in turn mostly limited by our fabrication approach. While it would be possible to make shorter devices, the expected residence times would be reduced, which would in turn make the experiment more challenging.

1 M. A. G. Zevenbergen, P. S. Singh, E. D. Goluch, B. L. Wolfrum and S. G. Lemay, *Nano Lett.*, 2011, **11**, 2881–2886.

Nongjian Tao commented: In the design of your nanochannels and measurements, how do you select or optimize the size of the electrodes, the spacing between the top and bottom electrodes, concentration of analytes and other parameters?

Serge Lemay answered: The device dimensions are largely limited by microfabrication constraints. The width of the channels is set by the resolution of the

photolithography method that we employ. Their height is set by the thickness of a sacrificial chromium layer that is etched away prior to measurements; while this can be controlled accurately, going below 40 nm tends to cause a much lower yield as imperfections near the edges and access holes cause short circuits between the electrodes. The length of the devices is very flexible (we've made devices with electrodes ranging from 10 microns to a millimeter) but for single-molecule experiments it was selected to yield longitudinal diffusion times ($L^2/2D$) on the scale of seconds (unfortunately such long events are missing in our experimental observations, as discussed in detail in the paper). The concentration is selected based on the device volume to yield less than one molecule in the device on average. Smaller volumes would simplify experiments as they would allow working at higher concentrations; this is an aspect that we are currently trying to address.

Olaf Magnussen commented: Did you assume in your theoretical treatment of the distribution times that these are given by the time of first return to the starting point in a 1D random walk? If yes, this may be problematic, since the same particle could reenter the channel directly (or after a short excursion of a few nanometers into the entrance hole) again, leading to a second event that is not statistically independent. Experimentally these events could not be resolved and would contribute to effectively longer events. This may significantly alter your statistics.

Serge Lemay replied: This is an excellent point and this behavior was indeed an important factor in our first published work on single-molecule detection.[1] In that case, the nanogap electrodes were imbedded away from the entrance of a one-dimensional channel, and it was likely that a molecule undergoing one-dimensional diffusion near the edge of the detection region would enter and exit repeatedly this region as suggested.

In the work presented here, a different geometry was employed in which the access holes into the redox-cycling region punch through the top electrode.[2] As a result, molecules coming back to the access holes after spending some time in the device will with a high likelihood diffuse back into solution. More technically, this corresponds to radiative boundary conditions at the inlets of the device, which yield a very low probability of exiting molecules returning into the device. We therefore do not expect that this behavior has a significant impact on the statistics described here.

We further note that 'composite events', in which a molecule repeatedly enters and leaves the detection region so fast that the train of events is interpreted as a single event, would lead to an excess of long events. The experiment however shows precisely the opposite tendency.

1 M. A. G. Zevenbergen, P. S. Singh, E. D. Goluch, B. L. Wolfrum and S. G. Lemay, *Nano Lett.*, 2011 **11**, 2881–2886.
2 S. Kang, K. Mathwig and S. G. Lemay, *Lab Chip*, 2012, **12**, 1262–1267.

Zhongqun Tian asked: Can you introduce or combine a micro-/nano-droplet technique with your micro-/nano channel system to make this new method even more powerful? If the electrolyte solution in the channel can be separated into many droplet with controllable droplet size and number of reactive species/

particle. In addition, the flow rate of the droplet and moving direction can be controlled to move into or leave the electrode pair region.

Serge Lemay responded: Thank you for this suggestion. It is certainly possible to combine our devices with droplet microfluidics since the materials and fabrication processes are compatible. This would make for very exciting experiments!

Wolfgang Schmickler remarked: These are elegant experiments. We believe that random walk theory holds for such processes, so the most interesting observations are deviations from this theory. You mentioned that traps may play a role. Could you please explain this in greater detail?

Serge Lemay replied: The distribution of residence times expected from random-walk theory is well defined and does not include any unknown parameters. Which diffusion coefficient to use, however, is subject to debate. It is our belief, based primarily on the interpretation of noise spectra at high concentrations[1–3] and of single-molecule current levels at low concentrations[4,5] that the effective diffusion coefficient is reduced due to reversible adsorption. Applying the same logic to single-molecule experiments, we would expect to see more 'long' events than we do in practice. This suggests the existence of sites in which the redox molecules can be trapped for an extended period of time.

1 M. A. G. Zevenbergen, P. S. Singh, E. D. Goluch, B. L. Wolfrum and S. G. Lemay, *Analytical Chemistry*, 2009,**81**, 8203–8212.
2 P. S. Singh, H.-S. Chan, S. Kang and S .G. Lemay, *J. Am. Chem. Soc.*, 2011, **133**, 18289–18295.
3 D. Mampallil, K. Mathwig, S. Kang and S. G. Lemay, *J. Phys. Chem. Lett.* , 2014, **5**, 636–640.
4 M. A. G. Zevenbergen, P. S. Singh, E. D. Goluch, B. L. Wolfrum and S. G. Lemay, *Nano Lett.*, 2011, **11**, 2881–2886.
5 S. Kang, A. F. Nieuwenhuis, K. Mathwig, D. Mampallil and S. G. Lemay, *ACS Nano*, 2013, **7**, 10931–10937.

Kristina Tschulik asked: The histogram in **Fig. 2b** shows a maximum residence time of 450 ms, while for a purely diffusional scenario the expected first passage maxima would be expected to be at lower values. Is the number of data points in the histogram statistically meaningful and if so does this observation hint towards the adsorption of $Fc(MeOH)_2$ at the electrode?

Serge Lemay replied: The appearance of a maximum in **Fig. 3b** is unexpected in any simple mechanism characterized by, for example, a single release rate. More likely our analysis is missing some of the shorter events with a duration around 0.5 s due to noise.

Sanli Faez said: To circumvent the lack of resolution for ultra-short events, you have based the analysis on events of a certain length or longer. If three or more electrodes were used instead of two, then correlative measurements could also resolve short paths. What is the prospect of performing these experiments with more than two electrodes?

Serge Lemay responded: This is absolutely correct and we have fabricated devices incorporating two or four electrode pairs for this purpose. The fabrication

strategy employed for the devices in the present article cannot be applied to such devices, however, due to geometry constraints. As a result, these devices have a relatively large 'dead volume' on either side of the active region, which decreases the single-molecule signal and introduces additional noise. We have not yet succeeded in obtaining single-molecule data with these devices.

Patrick Unwin commented: If you sum the residence time histograms in **Fig. 3**, you end up with a time-averaged fractional occupancy which is very close to what you would expect based on the concentration of redox species employed (between 0.05 and 0.06). However, events less than 450 ms are thrown away. This therefore suggests that the average occupancy is higher than might be expected. Is this additional evidence for adsorption on the electrodes? Theoretically, what is the total time of the < 450 ms events that are lost compared to those observed?

Serge Lemay responded: This was an acute observation, and after checking the calculation of the renormalization factor we have found an error. The curves were indeed inaccurately amplified. This will be corrected in the final manuscript and a footnote stating this change will be added.

Henry White remarked: This question regards adsorption of molecules in your thin-layer electrochemical cells. Have your ever put a molecule in one of these cells that does not adsorb? It appears to me that the observed 'adsorption' response may not be a chemical process but perhaps results from molecule entrapped in physical crevices, *etc.* Our laboratory has also observed a response in similarly constructed thin layer cells that suggests that adsorption of $Ru(NH)_6^{3+}$ on positively charged Pt electrodes.[1]

1 Q. Chen, K. McKelvey, M. A. Edwards and H. S. White, *J. Phys. Chem. C*, 2016, **120**, 17251–17260.

Serge Lemay answered: We have never seen a response that did not exhibit at least some degree of what we interpret as adsorption, either in the fluctuation spectrum or the time-dependent response. While this can be mitigated by temperature, potential or electrode modification, we have not found conditions that eliminate it completely. This is also true of $Ru(NH)_6^{3+}$, consistent with the observations of Chen *et al.* The question as to whether entrapment could be responsible is a fair one, but so far we have no direct evidence for this possibility: while we have not imaged devices post-measurement, metal films on which a sacrificial layer has been deposited and then etched using the same protocol as in actual devices did not exhibit additional roughness.

1 M. A. G. Zevenbergen, B. L. Wolfrum, E. D. Goluch, P. S. Singh and S. G. Lemay, *J. Am. Chem. Soc.*, 2009, **131**, 11471–11477.

David Fermin asked: Your analysis does not appear to include the possibility of ion pairing which is expected to be quite significant in the case of $Fe(CN)_6^{3-}$. Can you comment on whether this process can have an effect on the unexpected first-passage time statistics observed experimentally?

Serge Lemay responded: Ion pairing in the bulk would influence the effective diffusion coefficient of the species, but this is taken into account as we use diffusion coefficients measured in the same electrolytes using ultra-microelectrodes (UMEs). At the surface, however, there is evidence that the nature of the anion greatly influences the degree of adsorption, to the point where it follows the Hofmeister series.[1] Whether ion pairing is the correct theoretical framework to describe these species-dependent effects however remains unclear.

1 D. Mampallil, K. Mathwig, S. Kang and S. G. Lemay, *J. Phy. Chem. Lett.*, 20145, 636–640.

Andy Mount asked: If reversible adsorption were the process which leads to the observed value of D, D_{eff}, being lower in **Fig. 3** than the accepted value of D measured in solution, might one not expect a dependence of D_{eff} on the applied electrode potential (within the constraints that this potential should always be sufficient to ensure mass transport limited reaction of the redox species at the electrode), as the potential would be expected to affect the adsorption equilibrium position and the rates of the adsorption and desorption processes? Has this been observed?

Serge Lemay answered: We have indeed observed a dependence of the effective diffusion coefficient in measurements at high concentrations.[1,2] We did not perform a systematic study at single-molecule level, however. This is certainly a worthwhile experiment to attempt in the future.

1 P. S. Singh, H.-S. Chan, S. Kang and S. G. Lemay, *J. Am. Chem. Soc.*, 2011, **133**, 18289–18295.
2 S. Kang, K. Mathwig and S. G. Lemay, *Lab Chip*, **12**, 1262–1267.

Wolfgang Schmickler asked: I am surprised that the two redox couples, which are chemically quite different and also carry different charges, behave in such a similar way. I supsect that the trapping is not chemical, but a physical effect. Do you agree?

Serge Lemay responded: Yes, this is the most natural hypothesis. Note however that the apparent level of adsorption as revealed by noise analysis also depends both on the nature of the anion[1] and on modifications to the electrode surface.[2]

1 D. Mampallil, K. Mathwig, S. Kang and S. G. Lemay, *J. Phys. Chem. Lett.*, 2014, **5**, 636–640.
2 P. S. Singh, H.-S. Chan, S. Kang and S. G. Lemay, *J. Am. Chem. Soc.*, 2011, **133**, 18289–18295.

Richard Crooks asked: The electrolyte solution in your experiments is not degassed, and therefore the concentration of oxygen is about 8 orders of magnitude higher than the redox probe that you use for measurements. Accordingly, it seems likely that bimolecular reactions between the redox probe and oxygen will have a significant effect on measurements and hence conclusions from the experiments. Thoughts?

Serge Lemay responded: This is a very good point. Because of the small volumes involved, degassing in our setup would require building a gas-tight cell and we have not done this yet. We therefore cannot rule out interactions between our simple redox molecules and oxygen.

Richard Nichols said: Could you clarify any cleaning procedures for the nanochannels, this would appear to be not so straightforward as for planer disc gold or platinum electrodes which can be polished or flame annealed. 'Aggressive' oxidation/reduction (for example at high scan rates) have been used for *in situ* electrode cleaning. These can also dramatically alter surface micro/nano structure and increase surface roughness. Would such be suitable for an *in situ* cleaning? This may in itself be useful for increasing potential 'traps' and further addressing the issue of dwell time for the redox species on the surface. Also for macro electrodes and solution free redox couples the peak to peak separation in cyclic voltammograms is often useful for assessing the state of the surface and the reversibility of the electron transfer process. I realise that this could not be directly applied to such small cavity channels but are there any other diagnostic tests to assess the reversibility in nanochannels?

Serge Lemay answered: It is absolutely the case that a limitation of microfabricated devices is the inability to apply standard electrode polishing procedures. As an alternative, we routinely employ cyclic voltammetry in sulfuric acid to clean the electrodes following etching of the chromium sacrificial layer to create the nanochannel. Repeating this procedure after prolonged measurements (a few hours) yields non-ideal responses for the first scan, indicating that the electrode surface degrades over the course of the measurements. As an alternative to peak-to-peak separation for diagnosing electrode quality, mass transport in nanogap electrodes is also efficient enough to observe non-ideal, Butler–Volmer-like cyclic voltammograms even for very fast couples.[1] The apparent heterogeneous rate constant observed in such experiments does tend to decay over time on the scale of hours, but this can only be checked after the conclusion of single-molecule experiments since the introduction of sufficient concentrations for standard cyclic voltammetry make it impossible to return to ultra-low concentrations afterward.

1 M. A. G. Zevenbergen, B. L. Wolfrum, E. D. Goluch, P. S. Singh and S. G. Lemay, *J. Am. Chem. Soc.*, 2009, **131**, 11471–11477.

Frederic Kanoufi communicated: Based on the discussion, your device may behave as a nanocolumn of adsorption chromatography. Ferrocene has likely high affinity for the channel walls. One could get inspiration from separation science strategies: using a non-adsorbing compound, coating the channels with short-chain thiols to decrease adsorption and prefer partitioning, using a flow to optimize the solute residence time, using an optical readout such as fluorescence single-molecule analysis…

Serge Lemay communicated in reply: Thank you for the suggestions. We have indeed already partially implemented some of these approaches. First, we did observe a decrease in the level of adsorption at high concentrations upon modifying the electrodes with organothiols.[1] We have not yet attempted to combine this with single-molecule experiments, however. Second, we have built devices allowing convection of the sample along the channel, but it turns out that the PDMS material that we employed introduces too much contamination to allow sub-nanomolar measurements. Rectifying this problem is currently one of our main goals.

1 P. S. Singh, H.-S. Chan, S. Kang and S. G. Lemay, *J. Am. Chem. Soc.*, 2011,**133**, 18289–18295.

Sanli Faez opened a general discussion of the papers by Paul Bohn: You have mentioned that you could not image all nanopores together during the experiment, which explains why some of the electrically detected signal is not captured in the optical channel. What is the main limitation for measuring on a single nanopore? How about increasing the optical field of view to include the whole array of pores? What limits that?

Paul W. Bohn replied: The design of the nanopore array was undertaken to optimize two somewhat countervailing factors. In order to maximize electrochemical efficiency, we need small pore-to-pore spacing, which improves capture efficiency of molecules desorbing from a given nanopore by a neighboring nanopore. On the other hand, larger pore-to-pore spacings are required to allow the observation of a single nanopore by optical microscopy. The particular structure on which the published results were obtained was designed as a compromise between these two constraints. The pore spacing is small enough to yield good electrochemical capture efficiency but at the expense of not being able to resolve an individual pore.

Christine Kranz asked: All electrochemical data shown are obtained with a outer sphere redox species (hexaammineruthenium chloride), however most of relevant analytes in electroanalytical applications exhibit inner sphere behavior. Hence, how is the performance of the nanopore-confined recessed ring-disk electrode arrays for such applications? What is the effect of adsorption on the long-term performance?

Paul W. Bohn answered: We have conducted numerous additional experiments addressing the application of nanopore arrays to electrochemical detection, using, for example, $Ru(CN)_6^{3/4-}$, anthraquinone-2-sulfonic acid, ferrocene/ferrocenium, dopamine and ascorbic acid. These experiments establish that nanopore geometry is a key factor determining the magnitude of the electric double layer effect, as is the role played by the magnitude and sign of the charge of the redox-active species. Redox pairs exhibiting charges of different magnitude and sign show completely different response upon decreasing the supporting electrolyte concentration. Thus, the combined effects of ion accumulation and ion migration in these nanopore arrays overwhelm distinctions between inner and outer sphere redox processes.

Andy Mount asked: In a previous Faraday Discussions paper,[1] we reported significant changes in the voltammograms observed at our Microsquare Nanoband Edge Electrode (MNEE) array systems on reducing the ionic strength from hundereds of mM to tens of mM when utilising bands of 50 nm width. An additional current was observed in the diffusion-limited region of the voltammogram for two different redox species, ferrocenecarboxylate and hexaammineruthenium(III), which we attributed to the additional contribution from transport of these redox species due to migration. Have you observed similar effects in your dual electrode systems at lower ionic strengths and how does/would this complicate generator/collector analysis in your dual band systems?

1 I. Schmueser, A. J. Walton, J. G. Terry, H. L. Woodvine, N. J. Freeman and A. R. Mount, *Faraday Discuss.*, 2013, **164**, 295–314.

Paul W. Bohn answered: Yes, this is a very interesting phenomenon which we have also observed in our nanopore electrode arrays.[1,2] In fact, we are currently preparing a manuscript which clearly shows that the geometry of NEAs and the magnitude and sign of the charge on the redox species strongly affect both ion accumulation (permselectivity) and ion migration contributions to enhanced currents in NEAs. Our results confirm the screening of negatively charged species, in contrast to the accumulation of cations. Quantitative modeling and experiments in somewhat larger nanopores ($d \geq 500$ nm) has shown that at the lowest concentrations of redox species (1 μM), the resulting current enhancements can be assigned roughly 80% to ion accumulation and 20% to ion migration. Furthermore, the combination of ion accumulation and unscreened ion migration at low ionic strength provides additional sensitivity when coupled to redox cycling effect in our generator/collector system. In optimal cases redox cycling gives a ~50-fold current enhancement, while working without supporting electrolyte produces an additional factor of ~50–60, resulting in overall current enhancements as large as 3000 in favorable cases.

1 C. Ma, N. M. Contento and P. W. Bohn, *J. Am. Chem. Soc.*, 2014, **136**, 7225–7228.
2 C. Ma, W. Xu, W. R. A. Wichert, and P. W. Bohn, *ACS Nano*, 2016, **10**, 3658–3664.

Frederic Kanoufi remarked: I am wondering what is the sensitivity of your electrode array system, in terms of current detection/noise or highest frequency achievable. Actually the μA currents, provided by the large number of nanoelectrodes may not allow the use of a very fast acquisition rate which could be necessary for single entity detection.

Paul W. Bohn replied: The large currents referenced (μA range), which are exhibited in **Fig. 4** arise from two sources. One is the large number of nanoelectrodes (~10^5) in the array, another is due to the high concentration (1 mM) of redox species used. We operated under these conditions to illustrate the fundamental nature of the NEAs in GC and non-GC mode operation (sigmoidal I–V curves with little capacitance). For single entity detection, the concentration for single molecule occupancy in each nanopore is *ca.*1 μM. Under these conditions with ~10^5 nanopores we observe limiting currents of ~0.3 nA. Certainly, the large number of nanopores precludes observation of stochastic signals corresponding to single electron transfer events. Thus, in our more recent experiments, we work with smaller array sizes and at much lower concentration (1 nM, where the average electrochemical pore occupancy is 0.0011). Under these conditions, we observe highly correlated current fluctuations between the generator and collector electrodes. In addition, we observe smaller charging currents for the top collector electrode (**Fig. 5(B)**), which makes it possible to operate the experiment at scan rates up to 100 V s^{-1}.

Richard Crooks opened a general discussion of the papers by Andrew Ewing: In your model of the vesicle contacting the electrode surface, followed by a pore opening and subsequent rapid redox chemistry of the contents, how are the changes in the charge on the redox molecules compensated by the supporting

electrolyte? I ask because it seems like there is no way for the redox molecule and supporting electrolyte to interact across the bilayer membrane.

Andrew Ewing replied: At least for cell vesicles and decorated liposomes we think that proteins on the vesicle make space between the remaining membrane after pore opening to allow protons to diffuse out as they are oxidised and ions to diffuse in. The oxidation reaction is at the electrode where this would all occur, so I think it makes sense. We are working on this and will send you a manuscript when we finalize!

Wolfgang Schuhmann asked: You had to apply a quite high potential to oxidise dopamine. Does this potential change the adhesion of the vesicle (or the proteins inside the bilayer membrane) and is hence influencing the opening of the pore?

What did you mean with 'a non-electrified interface'? Each interface in an electrolyte is adapting to a certain potential.

Andrew Ewing answered: For dopamine amperometric detection it is standard to use about 0.7 V *vs.* AgAgCl so as to be on the wave plateau. This is not a very high potential. But, with the vesicles, 0.5 to 0.7 V across a 5 nm membrane is about in the range of field needed to electroporate a membrane. That is a main point of what we are stating here. We think the membrane is electroplated when the lipid bilayer gets close enough to the membrane to get to this field strength. The diffusional evidence suggests that the potential does not influence the adhesion of the vesicle to the electrode.

I cannot find where I used 'non-electrified' Otherwise, I agree.

Justin Gooding asked: The model of the membrane pores in the different liposomes and vesicles suggests that the size of the pore is related to membrane tension. What do you feel the role of the membrane viscosity is? Do you think adding cholesterol to make the membrane less fluid is worth exploring?

Andrew Ewing responded: Excellent idea. We have not explored membrane tension in this regard, but it has been discussed. It is difficult with vesicles to manipulate, but the cholesterol idea is a great one to try.

Jan Clausmeyer commented: There is a marked difference in the opening behavior between artificial liposomes decorated with peptides and chromaffin vesicles which carry a complex mixture of proteins. Is there already any information on how the nature of the proteins might affect the interaction with the electrode surface? How was the peptide selected? Does the electrode material matter for the vesicle opening mechanism, too?

Andrew Ewing responded: We only looked at the one peptide so far. In the paper we conclude though that the main difference is probably from the presence of the protein dense core in the vesicles that will inhibit diffusion. The electrode material needs to be studied more.

Patrick Unwin asked: I'd like to support the arguments made by Henry White about the location of the pore opening. Even if the pore opens at the top of the

impacting vesicle, the area of the collector electrode is enormous and there is a strong concentration gradient towards it because it acts as sink. I would have thought that the collection efficiency would be reasonably close to 1 and that the timescale and profile would be of the order that seen in the paper. Have you done the simulations for this case?

Andrew Ewing answered: I agree with the arguments for diffusion alone, but we have other arguments for the pore opening at the electrode *via* electroporation including the potential dependence which matches electroporation, simulations with the nano tip, the peptide result, we see pre-spike feet, *etc.* I will not say more here as we are preparing a separate manuscript and will send it to you later. But, fundamentally, on the diffusion argument you are correct and I was incorrect, except maybe the overall time, which we will address in the new manuscript.

Martin Edwards communicated: The numerical simulations presented below complement the calculations of characteristic diffusion times as discussed by Profs. Unwin and White and support that suggestion that the time for the current to fall upon vesicle opening and the amount collected is not sufficient on its own to suggest that the vesicle opens on the bottom.

The simulated flux-time curve presented (Fig. 1) is for the 'worst case' scenario where the vesicle opens with a pore on top. Despite this, essentially all of the contents is recovered by the electrode within a relatively short time (integral of flux equals molar content of entire vesicle = vesicle volume × concentration). It is interesting to note that the timescale is comparable with that shown in **Fig. 3** in the paper.

Also shown is a concentration plot taken at 2 ms (Fig. 2). The flux lines shown in white show how all of the flux from the vesicle is collected at the electrode.

Simulation details: Comsol Multiphysics 5.2a, diffusion coefficient 5×10^{-6} cm^2 s^{-1}, vesicle diameter 330 nm, pore opening diameter 33 nm radius, vesicle initial concentration 80 mM. Note: changes to simulation parameters will change the exact shape of the curve, but the collection efficiency is expected to be comparable.

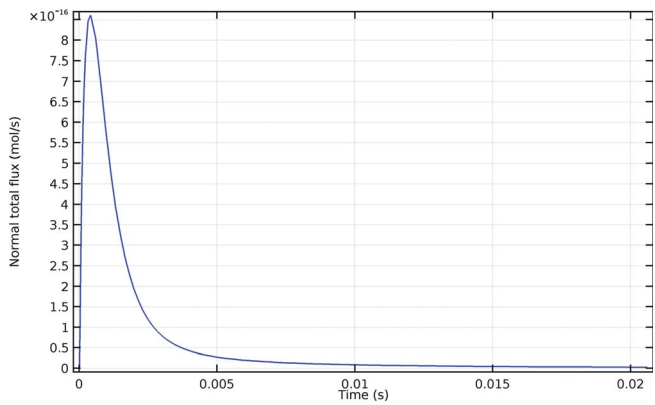

Fig. 1 Flux *vs.* time for release from a pore at the top of a vesicle.

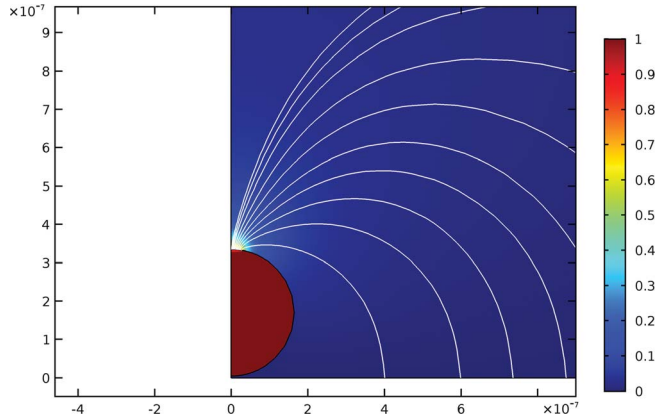

Fig. 2 Concentration of the molecule released from the vesicle (colour) and flux lines (white). Taken 2 ms after the start of simulations.

Andrew Ewing communicated in reply: I am surprised you publish this now, and we have discussed this in detail. It looks like the sim has a slower tail and it does not explain the differences between the peptide liposomes and the liposomes, which for a pore at the top should be the same. It also does not explain the potential dependence, the consistency with nano tip electrodes in and out of cells, and especially the presence of the pre-spike foot.

Christine Kranz opened the discussion of the paper by Lane Baker: I wondered about the set up for the experiment sketched in **Fig. 5a**. The positioning of the probe at the Ca^{2+} source seems to be rather cumbersome. Why wasn't the transport of Ca^{2+} through pores (as shown in **Fig. 8**) not used to demonstrate the functionality of the ICP-SICM probe?

Also what are the white spots visible in **Fig. 8b**?

Lane Baker replied: The setup is cumbersome, but quick. To be honest, we were rushing to meet the deadline for this submission, and the setup in **Fig. 5a** was much quicker to implement. As you point out, the setup in **Fig. 8** is a much better way to quantitate what is happening, and really demonstrate diffusion (or transport) measurements, and we have done that now also. In **Fig. 8b** the white spots are debris adsorbed to this particular membrane surface. We have since collected images absent of such features.

Christine Kranz added: In your paper you state that an increase in R_p is attributed to remaining cellular debris at the SICM barrel opening after the membrane patch was broken. Could you comment on whether such debris is affecting the imaging performance and in general if clogging of the second 'open' channel is an issue? Could you comment on success rate (some statistical data) on gigaseal formation on such dual barrels?

Lane Baker replied: The pipettes do drift some with time, but they generally never clog completely, and this is generally the case for SICM tips. The tips aren't

usually capable of making multiple consecutive patches. So far the imaging performance isn't such a big deal, but we don't have a lot of data on that yet (no studies of resolution). The gigaseal formation is ~50% of the time and a channel is present another ~50% of the time (these are rough numbers), but a fair degree of this is (1) user experience and (2) protein expression level in the cell lines. Expression level is related to transfection efficiency and time, as right of now we use transient expression, where cells stop expressing eventually.

Martin Edwards communicated: Scanning ion conductance microscopy is typically used in a distance modulation mode.[1] Using a lock-in amplifier to detect an alternating current gives a much more stable feedback mode, especially if there is drift in the current or one is working with smaller currents such as is inevitable with smaller probes.[2,3] Can you operate these membrane patch probes in distance modulated mode? I wonder whether the bilayers would be stable under mechanical stresses; would there be any artefacts due to, for example, capacitive currents due to fluid forces causing changes in the membrane area?

1 C.-C. Chen, Y. Zhou and L. A. Baker, *Annu. Rev. Anal. Chem.*, 2012, **5**, 207–228.
2 A. I. Shevchuk, J. Gorelik, S. E. Harding, M. J. Lab, D. Klenerman and Y. E. Korchev, *Biophys. J.*, 2001, **81**, 1759–1764.
3 M. A. Edwards, C. G. Williams, A. L. Whitworth and P. R. Unwin, *Anal. Chem.*, 2009, **81**, 4482–4492

Lane Baker answered: The probe configuration we show here is indeed stable with distance modulation. The last figure in our paper here is taken in AC mode, with feedback from the open barrel of the pipette. The patch remained intact throughout the course of scanning this image (a capacitive membrane test was performed before and after the image). This might give some clues as to where the patch is actually located on the probe, as if the patch were inside of the pipette it might be more stable under close probe surface distances that can be attained in with feedback imaging routines.

Richard Crooks asked: Your technique is based on detection through an ion channel immobilized on the capillary by patch clamping. How reproducible are the ion channels once attached to the capillary, what are the effects of irreproducibility on the conclusions that can be drawn from the experiment, and is there a non-electrochemical method to characterize the ion channel and its environment?

Lane Baker responded: Each probe is a unique tool, meaning that there is variability in the pipette, the patch and ion channel(s) in the probe. With that said, the conductance of the ion channels don't change a lot, but the number could change. Pipette geometry has an effect, although that may be a lesser effect for some aspects of sensing (because the ion channel is so small relative to the tip opening). With that in mind, each probe has to be calibrated if quantitative results are desired. Electron microscopy (especially STEM) is useful for characterizing the pipette. Previous work on larger pipettes has used TEM to look at patches in pipettes. These are hard experiments, but something on our radar.

Henry White asked: Ion channels frequently diffuse rapidly in lipid bilayers. I was wondering how this diffusion would affect imaging resolution where the ion channel is in a relatively large lipid patch suspended across the orifice of a pipette?

Lane Baker answered: Yes, this is definitely true, channels will diffuse. There is a difference here relative to synthetic bilayers, as 'real' bilayers from cells have components (*e.g.* cholesterol) that change diffusion properties, but it surely won't stop diffusion completely. Because we are imaging with the open barrel, the topographic resolution probably wont suffer much, but the 'chemical' resolution from the ion channel will be influenced. This also will depend on if the patch is an inside-out or outside-out patch, as the membrane area available to the channel(s) is likely different also, although we still have no real structural information on the geometry of the patched barrel. We have some plans to try and measure diffusion within the patch by holding the tip at a fixed distance and seeing if we can used diffusion of the channel to stochastically map the area under the patched barrel. That may or may not help define the extent to which diffusion influences this technique.

Paolo Actis commented: To achieve high resolution imaging there is a need for very sharp nanopipettes but it is very difficult if not impossible to excise a membrane from a sharp nanopipette. Novak *et al.*[1] proposed a method to precisely break the nanopipette tip to solve this issue. How are you planning to address the spatial resolution/selectivity issue?

1 P. Novak, J. Gorelik, U. Vivekananda, A. I. Shevchuk, Y. S. Ermolyuk, R. J. Bailey, A. J. Bushby, G. W. J. Moss, D. A. Rusakov, D. Klenerman, D. M. Kullmann, K. E. Volynski and Yuri E. Korchev, *Neuron*, 2013, **79**, 1067–1077.

Lane Baker replied: While SICM is surely capable of high resolution imaging, I'm not sure we will go after that aspect with these probes. The probes have fairly strict geometric requirements to allow the dual barrel approach shown here, and the *in situ* breaking method of Novak might be hard to implement here. We have decent resolution for finding larger locations at cell/tissue interfaces, and that is what we are targeting first. It is really interesting to think about using smaller probes in the future, we just aren't there right now.

Martin Edwards asked: Due to the way you produce the probe I assume that the number of pores you end up with is a stochastic quantity. Your data appear to show a single channel. How may pores do you typically get in your probe? And do you do anything to control this number?

Lane Baker answered: So far we haven't done anything to control the number, in general the protein expression level seems to be variable. We have looked at populations of channels in different patches and typically get 0, 1 or 2 open conductance states.

Zhongqun Tian opened a general discussion of the paper by Paul Bohn: I think the most important subject in the field of single entity electrochemistry could be

the single active sites, *e.g.*, single adsorption/reaction sites. It may be necessary to note that single-atom catalysts is becoming a new frontier in heterogeneous catalysis, focusing on single atoms anchored to metal oxide/metal surfaces. These reaction active sites are monodispersed at the surface and show very high activity and selectivity.[1,2] More importantly, two reports just published directly relate to electrocatalysis.[3,4] The next extremely hard target could be to characterize and thus understand structurally well-defined sites. Since the 1970s electrochemists have tried to use structurally well-defined single crystal surfaces to mimic the active sites because the atomic structure is the same periodically across the whole surface, which is easily characterized and simulated theoretically. However, people have realized that these single crystal surfaces cannot represent the whole active sites in real electrochemical systems well, *e.g.*, in electrocatalysis, electroplating, corrosion, because the single site includes the support/substrate. Preparing single entity active sites then well characterizing them one by one are a high challenge target. Vibrational spectroscopy may be a good diagnostic tool, such as tip-enhanced Raman spectroscopy (TERS). Could I have your comment on this?

1 X.-F. Yang, A. Wang, B. Qiao, J. Li, J. Liu and T. Zhang, *Acc. Chem. Res.*, 2013, **46**, 1740–1748.
2 J. D. A. Pelletier and J. M. Basset, **Acc. Chem. Res.**, 2013, **49**, 664–677.
3 P. Yin, T. Yao, Y. Wu, L. Zheng, Y. Lin, W. Liu, H. Ju, J. Zhu, X. Hong, Z. Deng, G. Zhou and S. Wei, Y. Li, *Angew. Chem. Int. Ed.*, 2016, **55**, 10800–10805.
4 S. Yang, J. Kim, Y. J. Tak, A. Soon and H. Lee, *Angew. Chem. Int. Ed.*, 2016, **55**, 2058–2062.

Paul W. Bohn replied: We agree that the preparation, characterization, and study of single-atom reactive sites is a 'holy grail' problem in (electro)chemical dynamics. Ideally, one could also study the reaction events one-at-a-time. These experiments are, as you appreciate, exceptionally difficult. From the outset, one may distinguish between two limiting systems: (a) those in which the special atomic sites are placed with structural regularity – and are thus relatively straightforward to find, but difficult to synthesize/fabricate; and (b) those in which the atomic sites are randomly placed – thus making the structures relatively easy to fabricate but the atomic sites difficult to locate for single-entity studies. We also agree that, realizing the caveats above, high spatial resolution vibrational spectroscopy (such as TERS) would be an excellent tool for such studies provided that the sensitivity is sufficient for the observation of the extremely limited number of reactants and products. In this regard, the construction of artificial nanostructures (not our nanopores necessarily, but other nanostructures allowing the volume to be focused on the single-atom site of interest) may provide a significant advantage.

Ashley Page directed a general discussion to the paper by Lane Baker: In the paper you deliberately over-express certain ion channels under the hope that a given membrane patch contains an individual ion channel of the desired type (Ca^{2+} gated K^+ channels/BK channels *etc.*). How often and to what extent do you see a convolution of the signal from native ion channels and how do you rule this out as a contributing factor in your experiments?

Lane Baker responded: We are using HEK cells that don't have a lot of other high conductance channels. But for sure, some are there. Because we know what

the conductance and the shape of the current–voltage response for the channels we've chosen to look at, we can make sure the probe responds in a manner that strongly suggests what we have at the tip. One advantage we have here is – so far – we aren't doing any new biology, so we have decades of results from real patch-clamp measurements to use for reference.

Nongjian Tao opened a general discussion of the paper by Andrew Ewing: It would be great to visualize individual particles (or vesicles) as they land and react on the electrode with an optical imaging technique. Of course, there are always important details that are impossible to 'visualize' experimentally. That's where theory (and your model) can play an important role.

Andrew Ewing responded: Thank you. We will think about it.

Richard Crooks opened a general discussion of the introductory lecture by NJ Tao: What is the source of the signal in the SPR image for *Cyt C* and why is the background signal so low?

Nongjian Tao replied: Both traditional and plasmonic cyclic voltammograms (CVs) show redox peaks of *Cyt C* adsorbed on an electrode, but the peaks in the plasmonic CV are more pronounced than those in the traditional CV. This is because the plasmonic image measures the optical polarizability difference between the reduced and oxidized molecules. In the case of proteins, *e.g.*, *Cyt C*, the difference is large because of the conformational change in the protein associated with oxidation and reduction, which leads to relatively large redox peaks in the plasmonic CV. The background current in the plasmonic CV arises from double layer charging (like the traditional CV), which is small because of surface passivation by a mercaptopropionic acid layer.

Patrick Unwin opened a general discussion of the paper by Paul Bohn: You presented some new data where the anti-correlated 'step changes' in the current of the generator and collector electrodes seemed quite long, of the order of a second or more. Given the high density of elements in the array and the concentration of molecules in solution, are discrete changes in the population of molecules in the device (loss/removal of a molecule) on that timecale reasonable? Have you run simulations for these experiments?

Paul W. Bohn responded: We are currently conducting detailed simulations of the behavior of these nanopore arrays, but do not yet have complete results. However, the observed times are reasonable considering a simple model based on freely diffusing molecules. The nanopore electrode arrays were patterned in a 20 $\mu m \times 20$ μm square array with an inter-pore (center-to-center) separation of 250 nm, giving an 80×80 array. The key feature affecting transport dynamics is that when a molecule escapes a nanopore, it is likely to be captured be a neighboring nanopore. Thus, in this simple picture a molecule is only lost when it escapes the entire array which would require a series of capture-hop-capture events. Diffusion over 10 μm would be of the order $t = x^2/2D = (10\ \mu m)^2/(2 \times 10^{-6}\ cm^2\ s^{-1}) = 500$ ms for all of the inter-pore diffusion events. When the molecule resides in a pore, the average duration time in single nanopore is about ~10 ms. Thus, ~40 captures

would be required to reach the edge of the array and escape, giving rise to an additional 400 ms. Thus, within the assumptions and simplifications of this picture, capture times of the order 1 s, as observed experimentally, are reasonable.

Bradley Thomas asked: In response to two different questions posed to you by other delegates you have said you never perform post-experiment analysis of your material. This would clearly be a worthwhile pursuit given the questions it would enable you to answer, so is there a fundamental reason for not doing so this given the breadth of techniques available?

Paul W. Bohn responded: There is no fundamental reason (beyond time and cost) that would prevent us from examining the nanopore arrays in cross-sectional SEM imaging *ex post facto*, and we appreciate the excellent suggestion. However, it is important to understand the limitations of such an experiment. First, the cross-sectional images will at most be able to reveal the structure of a few nanopores located proximal to one another. We could possibly get around this by sequentially moving and re-sectioning the device in the FIB. The other issue with respect to the optically-interrogated nanopores is that although we can identify the locale from which the optical data are acquired, it is difficult to pinpoint these pores exactly, so sectioning to reveal the state of these pores after the experiment would involve some uncertainty.

Michael Eikerling opened a general discussion of the paper by Andrew Ewing: **Fig. 6** in your article shows the different surface-functionalized vesicles considered in your study. The figure is used in an attempt to provide a mechanistic explanation of the observed rupture process. What role does the surface tension of the vesicle (that varies with chemical composition and surface structure) play in the process? Can this effect be explored systematically by varying the size of the vesicle and the chemical structure of chemisorbed species (using simpler surfactants)?

Andrew Ewing responded: I would guess that tension will play a role in the size of the pore that opens. I think this is a good idea to test the vesicle sizes and perhaps osmolality to look at this effect. I suspect, however, that the tension is the smaller affect *versus* the wettability and pore restrictions that are likely more from the functionality of the surface. Experiments like you propose will be needed to test this I think.

Patrick Unwin opened a discussion of all three papers by communicating: With small scale electrodes, the issue of contamination becomes increasingly problematic and I wonder whether each of you could comment on the significance of such effects in the experiments and systems you have described:

(a) For Andrew's studies on vesicle cytometry, it is well known that the oxidation of dopamine at high concentrations leads to the fouling of carbon electrodes[1] and as many electrochemical impacts are recorded in these studies and the 'electrochemical footprint' will be a bit larger than the projected area of the vesicle, does this lead to a deterioration of the electrode response over time? Could such effects contribute to the decay plots of frequency *vs.* time shown in **Fig. 3**?

(b) For Paul's studies on recessed ring-disk electrodes, how stable is the voltammetric response over time and how long can experiments be run?

(c) Could Lane make some comment on the advantages and disadvantages of SECM *vs.* SICM tips in terms of long term use and fouling effects?

1 A. N. Patel, S. Tan, T. S. Miller, J. V. Macpherson and P. R. Unwin, *Anal. Chem.*, 2013, **85**, 11755–11764.

Andrew Ewing responded: Actually, the projected area is defined by an area that is based on a twice the diameter of the vesicle. But, the vesicles are so small compared to the electrode we see no decrease over time during these experiments. Considering the overpotential and that we are doing amperometry, this might not be surprising. Also, this might also argue for the pore being at the electrode–vesicle interface. If this is the only place for messengers to escape, with high over potential, they will be trapped and oxidised at the small region of the electrode. If they are coming from the top, then the area affected by the response is much larger and could be fouled for future events. I think, however, this is less likely.

Paul W. Bohn responded: If the recessed ring-disk electrodes are characterized in full supporting electrolyte solution, the electrochemical signal is very stable – at least 100 CV scans (each scan around 10 s). In addition, the measurements are qualitatively reproducible across different sample conditions (*i.e.* draining and refilling the nanopore array) even over periods as long as a few months. This is quite fortunate, because the fabrication and conditioning of the arrays is non-trivial.

Lane Baker replied: For the question of SECM *versus* SICM tips for electrode fouling, there are two significant points. First, ease of preparation of SICM tips makes electrode fouling a less significant issue because less time is invested in the preparation of each individual tip (relative to SECM tips). Second, SICM pipettes have an advantage that they don't rely on faradaic electron transfer at the smallest, most crucial part of the probe, so they seem to be more resistant to fouling. SICM tips do change subtly in terms of ion currents when used for extended periods, but they can be used for extended periods in solutions of proteins without failure. SICM tips do clog at times, but not really to the extent one might imagine. I think once a passivating layer of whatever is in solution adsorbs to the glass wall, that likely mitigates further adsorption.

Venkateshkumar Prabhakaran opened a general discussion of the paper by Paul Bohn communicating: The diffusion of redox molecules in the nano pore array electrodes is also one of the important factor that determines performance, efficiency and sensitivity of redox cycling. Could we use electrochemical impedance spectroscopy in this study to estimate diffusion parameters and correlate to performance data?

Paul W. Bohn communicated in reply: Impedance spectroscopy is definitely possible, given the low noise floor ($\leq$ 20 fA) we routinely observe in our measurements. Although this paper and our previous papers on electrochemistry in nanopore arrays are focused on voltammetry, the diffusive transport of redox species can induce a Warburg impedance, from which (in principle) information

could be obtained regarding the diffusion of oxidant and reductant. We appreciate the question, because it may give rise to a route to measure hindered diffusion – a characteristic behavior of molecules in confined environments, such as our nanopores.

Richard Nichols opened a general discussion of the paper by Jens Ulstrup: When hemin is attached to the surface through the thiolated quadraplex the voltammetry is much sharper, while when it is physisorbed it is much less distinct. What is the reason for this? What is your model for the changes?

Jens Ulstrup replied: This observation was in fact somewhat unexpected. Physisorption around pH 7 is weak and gives generally poorly ordered adlayers. Both the voltammetry and the order as disclosed by *in situ* STM are much better at high (10–12) pH. Hemin marking of the quadruplex must, however, be around pH 7 to maintain stability of the quadruplex. The way we look at it, is that hemin is bound terminally to the quadruplex molecules by π–π stacking in well-defined configurations and that the quadruplex in turn is bound to the surface in coverages higher that hemin binding directly to the Au(111)-electrode surface. In addition the strong signals might then also originate in facile electron transfer through the (not so long) quadruplex molecule.

Nongjian Tao commented: Is there a way to characterize your DNA quadruplex and redox-modified quadruplex structures with a structural technique, *e.g.*, X-Ray?

Jens Ulstrup answered: The single-strand oligonucleotide used is not a commercial product but prepared and purified by the Nucleic Acid Center at University of Southern Denmark. We are keen to characterize the quadruplex structure further and have initiated steps first to use CD spectroscopy. I do not know how easy it would be to crystallize the samples, but NMR spectroscopy might be an alternative option.

Olaf Magnussen asked: You observed 2 different structures in your STM studies of the DNAzyme. Could you give the characteristic dimensions of those, *i.e.*, the line spacing and mesh width, respectively? How do these compare those to the size of 12G-qs and the DNAzyme? Do you see both structures also in the absence of hemine? Could hemin bind to to two neighboring 12G-qs and thus assist the formation of the observed small patches of aggregates in a more disordered matrix?

Jens Ulstrup responded: The mesh width was measured to be 1.6 ± 0.2 nm, which involves hydrogen bonding between two adjacent guanine bases. We can therefore say that the side length of the G-quartet is 1.6 ± 0.2 nm. The line spacing of Domain 2 in **Fig. 5B** was measured to be 1.5 ± 0.1 nm, which corresponds to the intermolecular distance between the two adjacent single strands of unfolded 12G-qs.

Both structures would most likely form also without hemin, but we are still working towards *in situ* STM visualization of hemin-free 12G-qs.

Hemin binding to two neighboring 12G-qs might be possible and would, presumably, then extend the linear structural units in Domain 2. This might also lead to small patches of aggregates, but there is no immediate evidence in the images that this is what is seen.

Robert Johnson said: At negative potentials *circa* −0.8 V *vs.* SCE, double-stranded DNA at the electrode surfaces can 'unwind' or 'denature' into its constituent single stranded components. See, for example, Johnson *et al.*[1] and Palecek.[2] A G-quadraplex is held together by Hoogsteen hydrogen bonding, and so I suspect when immobilized at the electrode it may be possible to unravel the quadraplex as the potential is scanned negative. Do you observe denaturation and/or structural changes to the quadraplex at negative potentials?

1 R. P Johnson, A. M. Fleming, Q. Jin, C. J. Burrows and H. S. White, *Biophys. J.*, 2014, **107**, 924–931.
2 E. Palecek, *Collect. Czech. Chem. Commun.*, 1974, **39**, 3449–3460.

Jens Ulstrup answered: The G-quadruplex voltammograms were reversible in the sense that successive scanning gave the same voltammetric features, with no indications of irreversible denaturation, but we deliberately stayed away from very negative potentials. This was to avoid interference from reductive desorption of the linking Au–S bond, which we have seen previously to pose challenges around neutral pH. We agree that we must be aware of G-quadruplex unwinding at very negative potentials.

Kylie Vincent commented: The voltammograms you show in **Fig. 2** show Au electrodes after hemin adsorption from dilute solution, but the STM measurements (**Fig. 3**) were made on electrodes prepared from much more concentrated hemin. Have you looked at the voltammetry of electrodes prepared from concentrated hemin solition? Can you be sure that the STM image reflects a single monolayer of hemin, rather than stacks of hemin?

Jens Ulstrup answered: The pH of the preparation solution for the hemin modified electrodes in **Fig. 2** and **3** are different. The hemin modified electrode and the quadruplex voltammetry in **Fig. 2** was prepared from 0.2 or 0.5 μM hemin in 5 mM sodium acetate at pH just above 7. The hemin modified electrode in **Fig. 3** (STM of hemin) was prepared from 10 μM hemin in 10 mM sodium hydroxide, pH 12. Both hemin solutions were diluted from 1 mM hemin stock solution prepared by dissolving hemin powder in sodium hydroxide at pH 12.

The voltammetry of the hemin modified Au(111) electrode in **Fig. 3** was recorded also at pH 12 and exhibits surface-controlled behaviour. The coverage of the hemin monolayer calculated from the voltammetric peak area is $(4.3 \pm 0.6) \times 10^{-11}$ mol cm^{-2}. The STM image in Figure 3 shows a highly ordered dense monolayer of hemin with a coverage of 1.7×10^{-10} mol cm^{-2}. Stacks of hemin molecules would have to be highly uniform to accord with such images. We are therefore sure that the STM images reflect a single dense monolayer of hemin and the voltammetry a high-coverage sub-monolayer.

Patrick Unwin asked: Have you tried to produce an adsorption isotherm of hemin on gold? I noted that only limited concentrations have been studied and

that when the concentration was doubled from 0.25 μM to 0.5 μM the amount of hemin adsorbed went up by about an order of magnitude, which would appear to suggest strong interactions between adsorbed hemin molecules.

Jens Ulstrup replied: Yes, this strong increase suggests strong interactions between the hemin molecules in the adsorbed layers. Strong interactions are also implied by the *in situ* STM images of the dense monolayers and may arise from hydrogen bonds between partially deprotonated propionate groups of the two hemin molecules in neutral solution. We have not produced a proper adsorption isotherm but I am sure that such an isotherm would offer interesting information regarding parameters of the interaction between the adsorbed hemin molecules.

Richard Nichols remarked: This question concerns **Fig. 2** and the cyclic voltammogram from the surface adsorption with the low concentration of hemin. Would square wave voltammetry help here in enhancing these peaks? Also is reductive desorption a possibility for characterising the adsorbed thiolated quadraplex species? Reductive desorption has often been found to be very useful for characterising adsorbed thiol species. Would the thiolate reductively desorb as a solution free species?

Jens Ulstrup replied: To the first part of the question, yes square wave (or differential pulse) voltammetry would be expected almost certainly to help improve the voltammetric sensitivity at low hemin coverage of the electrode and is therefore worth trying. To the second part, yes given that there would be conspicuous reductive desorption signals, such signals would offer useful additional characterization. We did this previously with some success for single- and double-strand DNA where we could determine the surface coverage and also distinguish between Faradaic and capacitive contributions to the reductive desorption signals. To the third part, since the G-quadruplex part is electrostatically highly charged, the molecule would most likely desorb as a solution free species.

Kylie Vincent asked: You mention that 'DNAzyme' activity has been demonstrated for the hemin–DNA G-quadruplex structures. Can you make use of this electrocatalytic activity to further characterise electron transfer rates through the DNA structure?

Jens Ulstrup answered: It is frequently (though not necessarily always) observed that redox metalloenzymes give no voltammetric signals in the absence of their substrate but strong electrocatalytic signals when substrate is added. We have seen this also at the single-molecule scale for the copper enzymes laccase[1] and nitrite reductase[2] using *in situ* STM. If no substrate is present (dioxygen or nitrite), no *in situ* STM contrasts of the protein molecules are seen, but when substrate is present strong molecular scale contrasts appear, as if a conduction channel through the protein is triggered on substrate binding. This effect also seems to be accompanied by notable 'swelling' of the enzyme (nitrite reductase) as disclosed by electrochemical AFM.[3]

Substrate binding to hemin bound to the G-quadruplexes may trigger both electronic and molecular structural changes in hemin. Whether this would

transmit into the quadruplex structure is hard to predict but something that we are in the process of looking into.

1 V. Climent, J. Zhang, E. P. Friis, L. H. Østergaard and J. Ulstrup, *J. Phys. Chem. C*, 2012, **116**, 1232–1243.
2 J. Zhang, A. C. Welinder, A. G. Hansen, H. E. M. Christensen and J. Ulstrup, *J. Phys. Chem. B*, 2003,**107**, 12480–12484.
3 X. Hao, . Zhang, H. E. M. Christensen, H. Wang and J. Ulstrup, *ChemPhysChem*, 2012, **13**, 2919–2924).

Sanli Faez opened a general discussion of the paper by Simon Higgins: Could measuring conductance *vs.* temperature serve as an extra indicator of the hopping mechanism that is suggested? Why are the conductance measurements as a function of length (*e.g.* in **Fig. 2**) limited to only 3 lengths? What is the limiting factor?

Simon Higgins answered: Certainly the measurement of conductance as a function of temperature could be useful additional evidence for hopping *vs.* tunnelling. However, it is worth introducing a note of caution here. In the case of conjugated oligoporphyrin molecular wires, we have found that although the conductance of some of these families of molecules is indeed temperature-dependent, DFT combined with non-equilibrium Green's function-based transport calculations established that this was not inconsistent with a tunnelling mechanism.[1]

The limitation here is simply one of synthetic chemistry. As oligoaryls get longer, their solubility generally decreases exponentially. The longest ligands used in this study were already at the limit of solubility to be useful as reagents for making their desired metal complexes. At the cost of greater synthetic complexity, it would be feasible to attach long alkyl chains to one or more of the aryl rings in longer analogues, to increase their solubility. But as this would also add another variable, namely the presence or absence of alkyl substituents, we decided that the time costs outweighed the likely benefits, and instead, we chose to make the alternative family of thioether-contacted molecules for comparison with the pyridyl-contacted examples.

1 G. Sedghi,V. M. García-Suárez, L. J. Esdaile, H. L. Anderson, C. J. Lambert, S. Martín, D. Bethell, S. J. Higgins, M. Elliott,N. Bennett, J. E. Macdonald and R. J. Nichols, *Nature Nanotech.*, 2011, **6**, 517–523.

Wolfgang Schmickler opened a general discussion of the paper by Jens Ulstrup: I was surprised to see that changing the solvent resulted in a completely different current–potential curve, from a Gaussian-like peak to a sigmoidal shape. In your model, changing the solvent changes the energy of reorganization, and perhaps the part of the potential that drops off between the electrode and the conducting center. This can deform the current–potential curve somewhat, but cannot result in a completely different form. I suggest that a sigmoidal form indicates that the electrode is blocked in a certain potential range.

Jens Ulstrup responded: The sigmoidal current–potential curve of the viologen in aqueous solution could be reproduced by a model resting on 'gated' electron transfer, *i.e.* pre-organization of soft twisting of the two halves of the molecule

from twisted to coplanar orientation leading to faster electron transfer. The current–potential curve would assume a Gaussian-like shape if twisting is prevented or no longer soft in the ionic liquid. We are in the process of addressing this issue further.

Kylie Vincent opened a general discussion of the paper by Simon Higgins: Could you comment on why the hit rate for the complexes is lower than that seen for 4,4'-bipyridine, and why the hit rate is higher for the thioether complexes compared to the complexes with N-donor ligands?

Simon Higgins replied: We are not sure about the reasons for the lower hit rate for the complexes, but it could be connected with the fact that 4,4'-bipyridine (under ambient conditions) is neutral, whereas the complexes are charged. Probably the thioether group has a higher affinity for gold surfaces than a pyridyl group and this accounts for the higher hit rate.

Patrick Unwin said: Could you give us an idea of how easy or difficult these measurements are for small molecules? What's the success rate? Is there an art to doing these experiments?

Simon Higgins answered: I suppose there is always an art to making measurements! In this case, I would say that it is easier to learn to make the measurements than it is to gain the experience to know when the experiment is working well before the data are analysed. It is worth noting that I regularly supervise undergraduate MChem students who both synthesise molecules, and then undertake metal | molecule | metal junction conductance measurements on them, in a project lasting one academic year where the student spends 50% of their time on project work. In that case, though, the measurements would be performed in ambient conditions, and the experiments in the paper, performed in an electrolyte environment under electrochemical potential control, are more tricky. It takes a good PhD or postdoc a few weeks of effort to learn to do those measurements reliably.

Nongjian Tao remarked: You describe two different behaviours in the conductance *vs.* potential plots. These data are from conductance histogram analysis. It might be interesting to trap a molecule between the STM tip and substrate, and then sweep the potential to see the potential dependence of the conductance for the molecule. This may allow you see things that are missing in the histogram analysis.

Simon Higgins answered: In fact, we did try to take I/V traces, because we hoped to measure the transition voltage of 6V6 under electrochemical control using Fowler–Nordheim type plots, to see whether these varied as a function of oxidation state. However, it proved impossible to collect meaningful data over the required range of gate voltages; the preamp saturated easily, perhaps due to a faradaic current following cracking of the wax coating at high bias, and at high overvoltages the I/V plots were unfortunately irreproducible.

David Fermin asked: In the presentation, you concluded that the conductance dependence on the overpotential is determined by the environment (electrolyte)

rather than the electronic structure of the molecule itself. The model used in your studies contains two parameters associated with this behaviour, the reorganisation energy ξ and the fraction of the potential experienced by the redox centre (λ). Could you describe how these two parameters compare in aqueous electrolyte and ionic liquids?

Simon Higgins replied: The molecules I discussed in the paper were only studied in ionic liquid, so I cannot comment on the relative values of λ and ξ in ionic liquid and aqueous electrolyte for them. The viologen molecule '6V6' showed the very broad 'off-on' behaviour in aqueous electrolyte but later, we found it showed a much sharper 'off-on-off' conductance–overpotential relation in ionic liquid, and on fitting these curves, we found the aqueous electrolyte behaviour was best fitted with values of $\lambda = 0.83$ eV, $\xi = 0.2$, whereas in ionic liquid the values were $\lambda = 1.3$ eV and $\xi = 1$.[1]

1 H. M. Osorio, S. Catarelli, P. Cea, J. B. G. Gluyas, F. Hartl, S. J. Higgins, E. Leary, P. J. Low, S. Martín, R. J. Nichols, J. Tory, J. Ulstrup, A. Vezzoli, D. C. Milan and Q. Zeng, *J. Am. Chem. Soc.*, 2015, **137**, 14319–14328.

Olaf Magnussen asked: The pronounced differences between the behaviour in aqueous electrolytes and ionic liquids are intriguing. In the paper you assign those to the short Debye screening lengths of the ionic liquids and effective structuring of the ionic liquid in the nano-gap junction. However, the difference in the screening by a typical double layer at 0.1 M concentration and an IL, which typically consists of rather large ions, should not be that different. The concrete interaction of the conducting molecule with the molecular components of the liquid may be more relevant for these effects.

Simon Higgins responded: It may well be the combination of the use of an ionic liquid and the very small nano gap involved in our experiments that contributes to the very significant observed differences. The layered structuring of ionic liquids at interfaces may be particularly effective at screening in the nano-gap junction. We have seen more effective charge screening (through the parameter zeta in the KU equation) for both the viologen (6V6) and pyrrole-tetrathiafulvalene (6PTTF6) systems (in the latter case, across both of its 2 successive redox state transitions). Ionic liquids have been shown to build clathrate cage structures around molecules in solution. If such were present in our systems this may also promote charge screening. Moreover, the nano-gap dimensions could create partially overlapping double layers, which may be more effectively mitigated in the 'charge dense' ionic liquids. Of course, ionic liquids do not have solvation shells to lose.

Kristina Tschulik remarked: Ionic liquids often contain impurities (like chloride or water) that remain after the synthesis procedure. Do you expect that such impurities might play a crucial role in your measurements?

Simon Higgins replied: We dry the ionic liquids as thoroughly as possible by heating in high vacuum overnight, and the ionic liquid is added to the dried electrochemical cell under argon; the STM measurements are taken in a controlled atmosphere chamber under argon. We also examine the potential

'window' of each batch after drying, using cyclic voltammetry, and have not seen any evidence for Cl^- oxidation in the batches we have bought.

Wojciech Nogala opened a general discussion of the papers by Dongping Zhan: It is clearly seen that a bilayer lipid membrane (BLM) effectively blocks hydrogen peroxide reduction on the BLM modified gold nanoelectrode (**Fig. 1**, curve 1). After injection of horseradish peroxidase (HRP) into the cell, a few hundred femtoampere current steps that are observed (**Fig. 2**), which are ascribed to hydrogen peroxide electroreduction catalyzed by single HRP molecules are unusually high. As calculated in the paper, a corresponding average turnover number of 620 000 s^{-1} is enormously higher than other literature reported turnover numbers for HRP. This suggest that the observed current steps are possibly caused by phenomenon other than direct wiring of active HRP molecules to the nanoelectrode. One possible mechanism is that the HRP molecules enter the bilayer lipid membrane creating channels for H_2O_2, which is directly reduced on the negatively polarized gold surface.

Another issue is the frequency of the current step appearance. Is this comparable with expected collision frequency of HRP molecules with the nanoelectrode?

Dongping Zhan responded: We don't know exactly what happens here and we prefer to believe it is caused by the electric double layer charging because it is always there and difficult to recover even after a long experiment. We don't think the other steps are caused by the leakage of H_2O_2 during the orientation of HRP in the lipid bilayer, because it is not random but in good regularity, from which we calculated the turnover number in an acceptable error range. The turnover number is much higher because we think it is closest to the practical bio-environment of single HRP electrocatalysis because of the bio-mimic membrane. By using a nanoelectrode, the collision frequency will be much lower than that oberved in the microelectrode. Adopting nanoelectrodes is good for decreasing the collision frequency and helpful to judge whether the collision is caused by one single molecule or a few molecules.

Kylie Vincent commented: How do the turnover frequencies you measure for HRP from the contact experiments compare to turnover frequencies measured for HRP films at macroelectrodes? Admittedly mass transport may be limited at macroelectrodes, but can be significantly improved at a rotating disk electrode. Have you made comparable experiments at a macroelectrode? It would be useful to include a cyclic voltammogram recorded at a macroelectrode modified with lipid bilayer and HRP for comparison. This would also enable comparison of the onset potential for catalysis to that observed in **Fig. 1**. It would also be useful to show control experiments using deactivated or inhibited horseradish peroxidase to confirm that the electrocatalytic currents rely upon active molecules of HRP contacting the electrode.

Dongping Zhan replied: Actually, we calculate the turnover frequency from the most probable current steps or the integrated charge of single collision incidents, which are obtained from the chronopotentiometry. Since the concentration of H_2O_2 is as high as 20 mM and the number of HRP is countable, mass transfer is

not the problem. The current step shows the limit of the catalytic capacitor of HRP. There are many reports on the direct electrochemistry of HRP on a macro-electrode. That is why we didn't do the experiments. It is a good suggestion to use an inhibitor to stop the catalytic experiment, which we didn't pay attention to before.

Wolfgang Schuhmann said: You had to apply a very low potential at the bilayer-modified electrode to see the assumed direct electrochemistry of horseradish peroxidase. However, it is known that the catalytic cycle of horseradish peroxidase is through an iron(IV) oxo radical cation at very high potentials. Hence, you need an overpotential of more than 1 V to transfer the electrons with the enzyme. Why did you have to apply such a huge overpotential?

Dongping Zhan replied: Although the two reversible peaks were observed in the direct electrochemistry of HRP,[1] the reduction of H_2O_2 catalyzed by HRP occurs at a potential region of (−0.1 V, −0.3 V) *vs.* the AgCl reference electrode.[2] During the reduction of hydrogen peroxide, we think the Fe(III) is reduced on the electrode which needs only a small overpotential.

1 J. Li and S. Dong, *J. Electroanal. Chem,* 1997, **431**, 19–22.
2 X. Liu, Y. Huang, L. Shang, X. Wang, H. Xiao and G. Li, *Bioelectrochemistry*, 2006, **68**, 98–104.

Wojciech Nogala said: According to **Fig. 1** in the paper (curve 2), H_2O_2 reduction in the studied system does not occur at electrode potentials higher (more positive) than *ca.* −0.2 V *vs.* Ag|AgCl. Direct electron transfer HRP-catalyzed electroreduction of H_2O_2 occurs at much higher potentials.[1] This suggests that H_2O_2 reduction in the studied system occurs directly on gold and the biocatalytic activity of HRP might play no role in the electrode process.

1 F. Chekin, L. Gorton and I. Tapsobea, *Anal. Bioanal. Chem.*, 2015, **407**, 439–446.

Dongping Zhan replied: This issue shows the importance of the reference electrode. However, in the experiment, to simplify the measurements, an Ag/AgCl wire is well adopted as the reference in bioelectrochemistry. Different publications have reported different 'experiment' potential. Thus, the calibration of the potential is very important. As shown in **Fig. 1**, the lipid bilayer can block the reduction of H_2O_2 very well. It is in the presence of the HRP that the current step is observed and the background becomes much smaller. We think HRP should play a role.

Kylie Vincent asked: It is not uncommon for redox enzymes to show higher catalytic rates on an electrode compared to their behaviour in solution assays due to effective direct electron transfer to the immobilised enzyme. However, the turnover frequencies for HRP that you measure seem unusually high. Can you comment?

HRP is not necessarily a poor choice of enzyme for these experiments, but it will be useful to carry out future experiments on other redox enzymes that are able to engage in direct electron transfer in order to see whether rate enhancements are observed more generally.

Dongping Zhan replied: We chose HRP necause we have some experience with HRP. It is a good suggestion to try other enzymes. The high turnover number might benefit from the bio-mimic membrane of the lipid bilayer.

Richard Nichols opened the discussion of the papers by Dongping Zhan and Jens Ulstrup: All three papers in this session have the commonality of electron transfer through molecular species. It would be useful to have a comparison of the electron transfer distances for each paper. The most complex system in this respect seems to be the paper on single molecule catalysis at redox enzymes where more than one layer is implicit, with a silane layer, a bilayer membrane and then the HRP docking and presumably orienting to this upper surface. Some comments and comparisons between the electron transfer distance would be interesting.

Dongping Zhan answered: The size of HRP is about 2 nm by 3 nm by 3 nm. And we think the thickness of the silane supported lipid bilayer is at the same scale. If the orientation of HRP in the lipid layer is correct, the charge transfer distance is about 1.0 to 1.5 nm. This is a reasonable distance in electrochemistry for out-of-sphere reactions. By the way, we have no evidence whether the memebrane is multiscale or whether there is internal communication between the adsorbed HRP molecules.

Jens Ulstrup replied: The quadruplex box length in the first of the three papers is about 0.7 nm if the distances between the G-quartets are roughly the same as between the base pairs in double-strand DNA. The length of the 6C-sulfur linker is about 1 nm. Assuming that the quadruplex box is vertically oriented or slightly tilted on the Au(111)-electrode surface, the electron transfer distance would then be about 1 and 2 nm for hemin bound at the end adjacent and remote from the electrode surface, respectively. The apparent conductance from **Fig. 5** is 0.18 nS or $2.3 \times 10^{-6} \times G_0$. The distance dependence could in principle be addressed by comparison with G-quadruplexes of longer box lengths.

The total length of the target transition metal complexes with aromatic ligands and linking groups in the second of the three papers varies from about 1.5 nm to about 3.2 nm, *cf.* **Scheme 1** and **Fig. 2** in the paper, *i.e.* in a certain sense in the same range as for the quadruplex. Notably, the aromatic linkers give both more than an order of magnitude higher conductance $(1.5–9.6) \times 10^{-5} \times G_0$ and a very weak distance dependence reflecting the two-step hopping mechanism of these molecules and the aromatic nature of the linking groups. The electron transfer distance in the multi-layer catalytic system addressed in the third of the papers is probably longer but harder to assess. The three layers on the modified Au-nanoelectrode are first octyltriethoxysilane, secondly egg lecitine, and third the enzyme horseradish peroxidase (HRP). The former layer thickness would be about 1.5 nm. Uncoiled lecithin would be about 3 nm long, but the degree of folding on the surface is not clear. The spatial extension of HRP is about 6 nm but it is not clear to what extent the enzyme is embedded in the lecithin layer. Overall, however, the composite multi-layer system appears to correspond to a significantly longer electron transfer distance than in the quadruplex and transition metal systems.

Kylie Vincent remarked: In different ways, the ordering of molecules on a gold surface is important in all of the three papers we are discussing here. Jens Ulstrup has identified different types of ordered domains in the hemin–DNA G quadruplex structures on gold using STM. Dongping Zhan is relying on ordering of a lipid bilayer membrane on a gold electrode to prevent access of peroxide directly to the electrode. Simon Higgins' experiments rely on the complexes aligning perpendicular to the surface, lying between the Au STM tip and the Au surface. This question is really addressed to Simon: to what extent do the complexes really align vertically between the tip and the surface, and does the distribution of break-off distances observed in your experiments result from a range of tilted orientations of the complexes? How does this complicate the interpretation of your results?

Simon Higgins replied: I didn't have time to exemplify in my presentation that we measure the break-off distance (the distance at which the metal | molecule | metal junctions break down), as well as the conductance of the junctions. The procedure has been described in our previous papers. This break-off distance should correspond with the length of the molecule. This does not depend upon the molecules being vertically oriented before junction formation because the molecules are lifted up as the tip–substrate distance is increased. The molecules may begin by lying flat on the surface, or may migrate into the tip–substrate gap as the junction forms. The molecules can either be adsorbed in a sub-monolayer state on the surface, or may be present in solution.

David Fermin opened a general discussion of the papers by Dongping Zhan: It remains unclear to me the nature of the so-called HRP activation which leads to the unusual voltammetric behaviour in **Fig. 1**. Could you elaborate on the origin of the step increase in the cathodic current at −0.3 V in the forward scan as well as the hysteresis and current loop observed in the reversed scan? You also mentioned that hydrogen peroxide reduction is effectively blocked at the modified nanoelectrode in the absence of HRP. However, this does not appear to be the case as there is a clear negative background current over large portion of the potential window.

Dongping Zhan responded: This is indeed a one-cycle voltammogram, in which the potential is held at +0.2 V for 6 s and then scanned negatively. This phenomenon is very reproduciable and we will provide a similar figure obtained with a different electrode in our revised manuscript. We didn't do the experiment beginning at −0.3V because we want to observe the HRP catalytic reaction from inactive status to active status. It is a good suggestion that we may try in the future. However, the collision incidents were recorded at a constant potential at −0.45 V, where the HRP is always active.

Actually, the hydrogen peroxide is blocked sufficiently by the lipid bilayer. Or else, the current will be 20-fold higher than the current observed in **Fig. 1**. We can observe the cathodic current from the blue curve in **Fig. 1**. It is the dramatic difference that makes the case for HRP catalysis convincing.

If the modofied nanoelectrode is kept polarized to a more negative potential, I am afraid that the bilayer will be detached.

Christine Kranz commented: The experiments were performed at gold nanoelectrodes exhibiting radii as small as 12 nm. The limiting current was calculated based on the equation for disc-shaped microelectrodes. However, considering the reported dimensions herein and the fact that no TEM images were recorded to evaluate the actual size and geometry of the electrode, the authors should comment on the uncertainty that is introduced for the presented data related to an erroneous shape and/or size of the electrode. Besides the electrochemical characterization, which complementary techniques have been used to evaluate the actual size and geometry of the nanoelectrode?

Secondly, Figure 1 shows the scheme of an HPR-BLM modified nanoelectrode. How was ensured that the enzyme is integrated in the BLM layer and not just adsorbed and stacked at the bilayer? How would this affect the turnover rate, and again, what is the influence on the accuracy of the reported values?

Dongping Zhan responded: As indicated in a response to a previous question, we accept that nanoelectrodes are controversial because they are too small to be characterized, easily broken and unstable during the CV. Frankly, we don't know the exact state of the nanoelectrode, we just judge its quality by ultra-high scanning voltammetry and calculate its apparent diameter using the steady-state limiting current. We think the nanoelectrode should be OK unless it is abused at extreme potentials either in the oxidation region or in the hydrogen evolution region.

We don't have evidence on the orientation state of HRP in the lipid bilayer. We believe that the turnover rate is dependent on the orientation from the individual diversity of single collision incidents. In future, *in situ* coupling techniques should be developed to obtain this information.

Patrick Unwin remarked: I would like to point out that measuring the size of nanoscale electrodes by voltammetry alone is somewhat ambiguous. One has to assume a planar electrode geometry, but the electrode could be recessed and (much) larger, or protruding and smaller. Better is to do approach curve measurements, as demonstrated, for example, by Takahashi *et al.*[1] Better still is to use transmission electron microscopy (TEM), which is very powerful for examining the geometry of nanopipettes, as demonstrated by Baker's group,[2] and which we have used to produce detailed mass transport models for nanopipettes.[3] TEM can also be used for the types of electrodes (filled nanopipettes) described here, as shown for example in **Fig. 1** in a recent paper by Minkyung Kang *et al.* on nanoscale electrochemical imaging.[4] In fact, Macpherson and coworkers were using TEM more than a decade ago to characterise nanoscale electrodes integrated into AFM tips.[5]

1 Y. Takahashi, A. I. Shevchuk, P. Novak, B. Babakinejad, J. V. Macpherson, P. R. Unwin, H. Shiku, J. Gorelik, D. Klenerman, Y. E. Korchev and T. Matsue, *Proc. Natl. Acad. Sci. U. S. A.* , 2012, **109**, 11540–11545.
2 L. Zhou, Y. Zhou and L. A. Baker, *Electrochem. Soc. Interface*, 2014, **23**, 47–52.
3 D. Perry, D. Momotenko, R. A. Lazenby, M. Kang, and P. R. Unwin, *Anal. Chem.*, 2016, **88**, 5523–5530.
4 M. Kang, D. Momotenko, A. Page, D. Perry and P. R. Unwin, *Langmuir*, 2016, **32**, 7993–8008.
5 D. P. Burt, N. R. Wilson, J. M. Weaver, P. S. Dobson and J. V. Macpherson, *Nano Lett.*, 2005, **5**, 639–643.

Wolfgang Schuhmann commented: This comment is related to the comment by Pat Unwin concerning the easy characterisation of nanotips using TEM. Unlike in the case of capillaries, in the case of nanoelectrodes, charging by touching the electrode may destroy the electrode tip (see paper by S. Amemiya *et al.*).[1] Hence, frequent insertion of a nanoelectrode into a TEM may not always be so easy for in-depth characterisation of the tip.

1 N. Nioradze, R. Chen, J. Kim, M. Shen, P. Santhosh and S. Amemiya, *Anal. Chem.*, 2013, **85**, 6198–6202.

Dongping Zhan replied: Nanoelectrodes are controversial because they are difficult to prepare and characterize, fragile and easily broken, and the metal nanowire is unstable during the experiments. However, if nanoelectrodes are used in a proper way, it can provide convincing information over a certain period. Even if there are some defects, it depends on what kind of electrochemical reaction is being investigated. For example, leakage is the main problem for nanoelectrodes. However, it may have little effect on experiments under ultrahigh scanning rate because, due to the high resistance and capacitance in the ultra-narrow leakage layer and, thus, the high time constant, it has no time to response the ultra-high voltage scanning ($> 100\ V\ s^{-1}$) or ultra-fast voltage pulse (> 1 GHz) . We don't know what exactly happens in SEM experiments. The strong electron beam may destroy the nanoelectrode directly, especially a very small one. As we know, focused electron beam is a very important microfabrication method.

Shengli Chen opened a general discussion of the papers by Simon Higgins: In Prof. Higgins' paper, the electron transfer mechanism has been distinguished into tunneling and hopping mechanisms respectively. From my understanding, the hopping electron transfer should be also through a tunneling process which occurs over relatively large distance and requires orbital overlapping. So, what's the intrinsic difference between tunneling and hopping transfer here?

Simon Higgins replied: Yes, in the Kuznetsov–Ulstrup model, the electron has to tunnel from the electrode to the redox site in the molecule, and then again from the redox site to the other electrode. This is distinct from the situation where the frontier orbitals are far removed in energy from the Fermi energies of the electrodes (*e.g.* for molecules like alkanedithiols, or for short conjugated molecules), where the electron can then only get from one electrode to the other by tunnelling across the molecule. The first case is incoherent to a degree depending upon the extent to which the molecule can relax after the first tunnelling process; the second case is coherent.

Venkateshkumar Prabhakaran communicated: Ionic liquids are interesting and promising electrolytes in many electrochemical technologies as most of them do not need solvents and so avoid many side reactions caused because of them. An interesting property of ionic liquid is that it has differential double charging and formation of the solvated clusters with active species of interest during forward and reverse scanning due to the asymmetry of ion sizes. These properties makes them very difficult to model the interfaces comprising ionic liquid. Dissimilar double layer charging could significantly change the redox activity of

the conjugated molecules. Alexei Kornyshev *et al.* have published a series of papers on the double layer charging of ionic liquids. My question is if this property has been considered during studies, and whether these properties have been studied separately both in oxidation and reduction scans.

Simon Higgins communicated in reply: In our measurements, we hold the potentials of the two working electrodes (tip and substrate) fixed while collecting data; these are not cycling experiments. However, with a suitably long-lived junction, perhaps using strongly-bonded contacts like thiolates (Ar–S–Au) or direct Au–C bonds rather than 4-pyridyl groups, it may be possible to measure junction conductance while cycling the electrode potentials with a fixed bias, hoping that the junction lives long enough, although we haven't tried this.

