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ChemComm
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Electrochemical measurement of the interaction of *Crotalus adamanteus* venom with DMPC vesicles

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Received (in Cambridge, UK) 7th May 2004, Accepted 25th June 2004
First published as an Advance Article on the web 5th August 2004

Electrochemical measurements of large unilamellar vesicles encapsulating potassium ferrocyanide showed that the lysis of vesicles by *Crotalus adamanteus* venom could be measured showing both time and concentration dependent responses.

This communication describes a method for studying the lysis of artificial lipid bilayer membranes by action of the venom from *Crotalus adamanteus* (Eastern Diamondback rattlesnake). A redox probe, potassium ferrocyanide, was encapsulated in large unilamellar vesicles of di-myristoylphosphatidylcholine (DMPC). The lysis of the vesicles by the venom was measured electrochemically by recording the peak oxidation potential of the redox probe. Time dependent and dose dependent responses were measured. Control measurements in the absence of Ca^{2+} showed no lysis of vesicles. The venom was obtained from Sigma-Aldrich UK.

The venom of the Eastern Diamondback rattlesnake contains a complex array of enzymes and peptides including both phosphodiesterase I and phospholipase A2 (PLA2). A bite from such snakes results in haemolysis and tissue necrosis as a result of cell membrane lysis by enzymes, including phospholipase A2 (as well as other effects).[1] Lipid vesicles have been of general interest for many years now, as they can be considered as free floating biomimetic lipid membranes. Thus they can be used as model bilayer systems for studying peptides and proteins (for example) in cellular bilayer membranes.[2]

A number of studies of enzymatic action on lipid vesicles have been detailed in the recent literature, including an interesting study by Xu and Cheng, whereby streptolysin (a pore forming bacterial toxin) action on electrode immobilised large unilamellar vesicles filled with potassium ferricyanide was measured electrochemically.[3] However sensitivity in such surface bound systems is lower due to the relatively low concentration of surface adsorbed vesicles compared with the bulk system used in this study. Others have studied real time lysis of giant unilamellar vesicles by fluorescently tagged phospholipase A2.[4] Recently reported work by Oloffson *et al.* detailed a Quartz Crystal Microscope (QCM-D) study of the formation of POPC lipid bilayers on SiO_2 surfaces and the interaction with phospholipase A2, resulting in the destruction of the bilayer.[5] A loss of lipid film mass at the surface was measured after PLA2 action on the film.

In this study, unilamellar DMPC (Avanti Polar Lipids) vesicles were made by dispersing DMPC in pH 7.4 phosphate buffered saline (PBS) and 50 mmol dm^{-3} redox probe, extruding through 100 nm diameter polycarbonate membranes (Avestin Lipsofast) and concentrating by centrifuging at 4 °C (below the phase transition temperature of the lipid) for 6 min at 1000 rpm centrifuge, followed by dilution in PBS–$CaCl_2$.[6] The centrifugation and wash steps were carried out twice. The change in concentration of redox probe not encapsulated by the vesicle membrane was measured electrochemically (*i.e.* probe which has free access to the electrode).

A freshly polished glassy carbon electrode was used in a BAS thin layer cell with a downstream silver chloride reference electrode. Measurements were made on an Autolab PGSTAT12 potentiostat. See† for experimental details. The concentration of the redox probe is related to the peak height. The peak current is proportional to the concentration of redox probe, provided other peak parameters remain constant. Measurements were made at 23 °C, approximately the phase transition temperature of DMPC.

The first experiment involved making measurements of current with time before and after addition of venom to the concentrated vesicle dispersion, giving a final lipid concentration of 1.6 mg ml^{-1} (used throughout) and a venom concentration of 2 mg ml^{-1}. Calcium chloride in PBS was introduced to give a Ca^{2+} concentration of 1.6 mmol dm^{-3}. In all experiments the vesicles and venom were mixed outside of the cell, just prior to injection and measurement in the thin layer cell. Fig. 1 illustrates the change in oxidation current of the redox probe as it is released by action of enzymes in the venom.

Two control measurements were carried out in order to assess the effect of natural vesicle rupture not mediated by venom. Vesicles were introduced into the thin layer cell and measured every five minutes for twenty-five minutes. No change in peak current was measured, showing the natural rupture of the vesicles and leakage of redox couple through the lipid bilayers were not significant. The experiment was also performed with venom introduced, but in the absence of calcium. Calcium mediates the action of enzymes believed to be important in cell lysis, particularly PLA2. No change in peak current was again observed, confirming the role of calcium and suggesting that the principal mechanism of membrane lysis in this experiment may be the hydrolysis of 2-acyl ester bonds of the L-*R*-3-*sn*-glycerophospholipids.[7] Calcium is believed to mediate attachment of PLA2 to lipid membranes.[8]

Having established that it is possible to measure the effect of snake venom on lipid vesicles using this method, a concentration of venom *vs.* electrochemical response study was performed to determine whether the system could potentially act as a crude sensor of membrane lysing enzymes. The same experimental procedure was used as previously described, but with varying concentrations of venom.

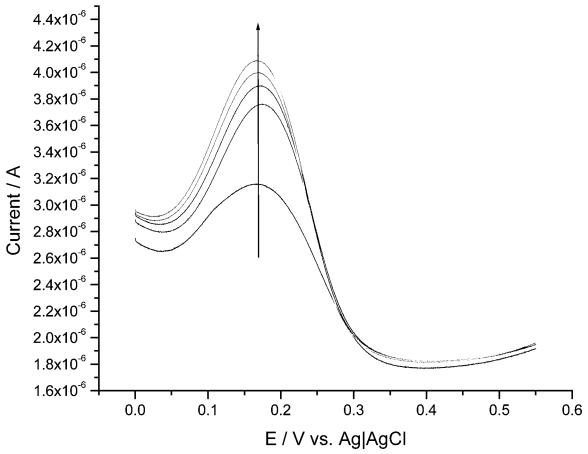

Fig. 1 Time dependence of lysis of vesicles by venom. The baseline curve refers to measurement of PBS in the cell, vesicle base line is the vesicles just prior to addition of the venom. Measurements made at 0, 5, 10, 15, 20 and 25 minutes following introduction of venom to vesicle suspension. Lowest peak height curve is vesicle suspension before introduction of venom.

DOI: 10.1039/b406799a

The relationship between peak current and concentration of snake venom is shown below. Measurements were made after 10 minutes interaction between the venom of vesicles outside of the thin layer cell. After 10 minutes the vesicle–venom dispersion was quickly injected and the differential pulse measurement recorded. The peak height increased as a function of venom concentrations between 0.8 and 8 mg ml^{-1}. The peak current for venom at 16 mg ml^{-1} is not significantly different from the value at 8 mg ml^{-1}. The current measured at 0 gram venom concentration is a result of residual redox probe left in the vesicle dispersion, but not encapsulated by the lipid membrane.

The shape of the graph can be understood by estimating the change in concentration of redox probe when all the vesicles are ruptured. The most straightforward way to do this is to consider a uniform size population of vesicle spheres with an outer diameter of 110 nm and an inner diameter 100 nm assuming a bilayer thickness of 5 nm.[6] If an average lipid head group area of 60 $Å^2$ is assumed, and the lipids are highly packed in a bilayer configuration[9] then it can be calculated that 1.15×10^5 lipids make one vesicle, of internal volume 1.66×10^{-16} ml. Given that the molecular weight of DMPC is 678 g mol^{-1} it can be shown that a dispersion of lipid of 1.6 mg ml^{-1} contains 1.24×10^{13} vesicles with a total internal volume of 6.5 μl (containing 50 mmol dm^{-3} redox probe). Hence, total rupture of all vesicles will change the concentration of redox probe from background to 0.3 mmol dm^{-3} in the electrochemical cell. Separate calibration measurements of the electrochemical cell showed that a concentration of 0.3 mmol dm^{-3} redox probe resulted in a peak height of 10 μA (for a differential pulse voltammogram with identical pulse parameters as the main experiment).

The above calculation, although based on a number of assumptions, serves to provide an order of magnitude estimate of the total change in redox probe concentration (and hence peak current) if all vesicles are lysed. The magnitude of the current increase is close to that experimentally determined and shown in Fig. 2. This suggests that the flattening out of the response observed in Fig. 2 is likely to be a consequence of lysis of all (or the majority) of vesicles in the dispersion.

The ultimate aim of this work is to use such redox probe filled vesicles as devices for amplifying some biochemical response (for example pore activation in a membrane) to give an easily measurable electrochemical response. For example, in principle, a single interaction of a pore forming peptide with redox probe filled vesicle could give a large change in redox probe concentration outside of the vesicle, which can then be easily measured. Moreover, the relative ease by which specific protein receptor molecules can be immobilised in vesicles provides the possibility to create new biosensors. This approach has been partially demonstrated by the recent work of Cheng in reference 10.

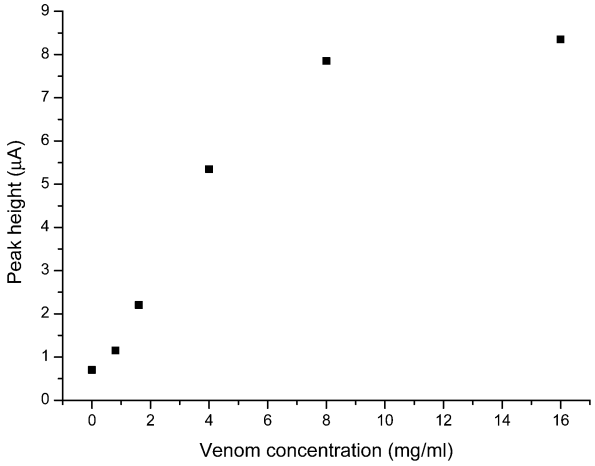

Fig. 2 Effect of venom concentration on peak current of redox probe. All measurements made at 10 minutes after venom introduction.

We would like to thank the Royal Society for an equipment and consumables grant that supported this work.

Notes and references

† Measurements were made on a 6 mm diameter glassy carbon electrode, separated from a stainless steel counter electrode by a 40 μm thick gasket. Initial potential 0.00 V *vs.* Ag | AgCl. Electrode conditioned at 0.00 V for 60 s prior to measurement. End potential 0.55 V. Scan rate 3 mV s^{-1}. Modulation amplitude 50 mV. Modulation time 0.04 s, interval time 0.1 s. Peak heights determined by fitting software (Autolab).

1 B. S. Gold, R. C. Dart and R. A. Barish, *New Engl. J. Med.*, 2002, **347**, 347.
2 M. Davidson, M. Karlsson, J. Sinclair, K. Sott and O. Orwar, *J. Am. Chem. Soc.*, 2003, **125**, 374.
3 D. Xu and Q. Cheng, *J. Am. Chem. Soc.*, 2002, **124**, 14314.
4 S. A. Sanchez, L. A. Bagatolli, E. Gratton and T. L. Hazlett, *Biophys. J.*, 2002, **82**, 2232.
5 L. G. M. Olofsson, M. E. M. Edvardsson, P. Delsing and B. Kasemo, *Sens. Actuators, B*, 2004, **97**, 313.
6 E. Kalb, S. Frey and L. K. Tamm, *Biochim. Biophys. Acta*, 1992, **1103**, 307–316.
7 S. Chen and H. D. Abruña, *Langmuir*, 1997, **13**, 5969.
8 S. A. Farber, E. S. Olson, J. D. Clark and M. E. Halpern, *J. Biol. Chem.*, 1999, **274**, 19338.
9 N. Kucerka, M. A. Kiselev and P. Balgavy, *Eur. Biophys. J.*, 2004, **33**, 328.
10 Q. Cheng, S. Zhu, J. Song and N. Zhang, *Analyst*, 2004, **129**, 309.