

Electrochemical Investigations of the Voltage Dependent Channel Opening Properties of a Gramicidin Analogue.

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Abstract:

Scanning Electrochemical Microscopy (SECM), using ion amperometry and chronoamperometry was used to investigate the comparative channel-forming properties of gramicidin A (gA) and a synthetic analogue gram-2-(nicotinamidyl)ethyl carbamate (gAN). Chronoamperometric experiments were conducted to determine voltage switching properties of gAN using Eu^{3+} , Tl^+ , K^+ ions. SECM experiments were conducted with two ions: permeant K^+ ions and the larger $(\text{CH}_3)_4\text{N}^+$ ion which blocks the channel. The switching potential for gAN, at which the channel allows flux monovalent cations, is shown to be potential and pH dependent and marginally temperature dependent.

Keywords:

Voltage-gated ion-channel, gramicidin, scanning electrochemical microscopy (SECM), pH, temperature, bilayer lipid membrane (BLM), ion amperometry, chronoamperometry.

Introduction:

The study of the interaction of channel forming synthetic and natural peptides within phospholipid membranes is an area of increasing interest.^{1,2,3} The pore-forming mechanisms and ion-selectivities of several peptide antibiotics have been extensively investigated using a variety of techniques, amongst the most prevalent of which are fluorescence microscopy, FT-IR, electrophysiology, AFM and NMR. The dynamics of charge-transfer in bilayer lipid membranes (BLMs) is usually probed by patch-clamp amperometric microelectrodes to probe ion-fluxes in biological membranes.^{4,5,6}

In this work we initially used chronoamperometric measurements, where a gAN ion-channel porated membrane was inserted on a gold working electrode (WE) surface (*vide infra*) and the potential was switched back and forth from an initial potential at which the ion-channel was closed to a step potential, where the electrode was held at the channel opening potential for a step time, to ascertain the voltage dependent channel forming properties of gAN. Following these studies scanning electrochemical microscopy (SECM) using ultra microelectrodes was used for further investigations.

The development of scanning electrochemical microscopy (SECM) techniques in the late 1980s by Bard and his co-workers,⁷ resulted in several publications on the use of this technique in probing the kinetics of ion-transfer (IT)^{8,9,10} and electron-transfer (ET) processes^{11,12,13,14,15,16} on monolayers at the interface between two immiscible electrolyte solutions (ITIES). Tsionsky, Bard and co-workers¹⁵ used SECM to probe the kinetics of ion-transfer processes on the surface of BLMs using approach curves. Amemmiya and Bard used voltammetric ion-selective microelectrodes to study K⁺ transfer through gramicidin channels in horizontal BLMs prepared by the brush technique.^{8,10} Mauzeroll, Buda and Bard¹⁷ used SECM to monitor the transport of thallium (I) ions across gA half channels inserted in a dioleoylphosphatidylcholine (DOPC) monolayer supported on a thallium amalgam hanging mercury drop electrode (HMDE) and obtained an apparent rate constant of $k_{het} = 2.8(\pm 0.1) \times 10^{-4} \text{ cm s}^{-1}$.

Gramicidin A (gA), a naturally occurring peptide antibiotic of sequence formyl-V-G-A-D-L-A-D-V-V-D-V-W-D-L-W-D-L-W-D-L-W-ethanolamine is a well-characterised membrane-active peptide. In membranes, as a result of the unusual alternating sequence of D- and L-amino acids, the peptide *is able to adopt* a $\beta^{6,3}$ right-handed helical conformation.¹⁸ The monomer is approximately 10.5 Å in length, less than the diameter of a single lamella in most membranes. Cation-selective channels are formed when gA monomers diffuse laterally in the membrane and form head-to-head (N-terminal-to-N-terminal) dimers. Previous studies have demonstrated that modification of the C-terminal ethanolamine with carbamate groups produces channels with modified conductance properties. In particular, single molecules of gramicidin A-ethylenediamine (gE), in which the terminal amino group is positively charged under physiological conditions, showed multiple conductance levels when studied by patch clamp techniques.²⁶ In the open form, gE displayed a maximum conductance that was approximately half that of gA, with additional conductance levels that were ~90%, ~50% and ~40% of this

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maximal value. These conductance levels were attributed to *cis/trans* isomerism of the carbamate group, with the positively charged ammonium group obstructing the channel entrance and/or exit in the *cis* isomer, but lying away from the channel axis in the *trans* form. The four possible *cis/trans* combinations were then responsible for the four observed conductance levels. It was additionally observed in control experiments, that the positively charged group was essential for production of this effect. We reasoned therefore, that the nicotinamide derivative gAN was a potentially interesting target, as in the oxidised form, the molecule would possess a positive charge in the same location as gE, and should therefore display similar conductance properties. In the reduced form however, this charge would be removed, and the channel should conduct, providing a means for producing redox-controlled channel activity. In effect gAN should provide a means of very precise regulation of the ion-channel at the redox potential of the nicotinamide moiety in contrast to gE where the ion-channel permeability varies with the four possible *cis/trans* combinations.

Ion Amperometry

A biological membrane inserted with an ion channel can be compared to an electric circuit. The dielectric constant is a measure of the relative membrane capacitance which is related to the capacitance $C = Q/E$ where Q is the charge on the capacitor and E the potential. The lipid membrane has a dielectric constant, $\epsilon \sim 2.0$ compared to ~ 80 for water at room temperature¹⁹. Insertion of gA creates a more polar environment, increasing the dielectric constant and allowing passage of hydrophilic cations. The effective dielectric constant, ϵ_{eff} , is assumed to be ~ 20 .²⁰

When a porated insulating BLM membrane is inserted between two ionic solutions, the ionic current is limited by the transfer of permeable ions across the membrane.²¹

The difference between the concentration of cations on either side of the membrane induces a diffusion flux, J , of ions which is dependent on diffusion coefficients, D , in the ion-channel and the concentration gradients on either side of a membrane porated with ion-channels and the potential gradient:

$$\Delta J_i = -\Delta D \left(\frac{\partial \Delta c_{i(1-2)}}{\partial x} \right) = v_i + \frac{d\tau_i}{dt} \quad (1)$$

where ΔD is the difference in diffusion coefficients of the permeable cations in the

aqueous phase and in the membrane ion channel, v_i is the ion transfer rate and τ_i is the concentration of ions on the membrane. The charge per unit time is the current, which splits into capacitive and a Faradaic term.²²

$$i = \sum z_i F A J_i = \sum z_i F A v_i + \sum z_i F A \left(\frac{d\tau_i}{dt} \right) \quad (2)$$

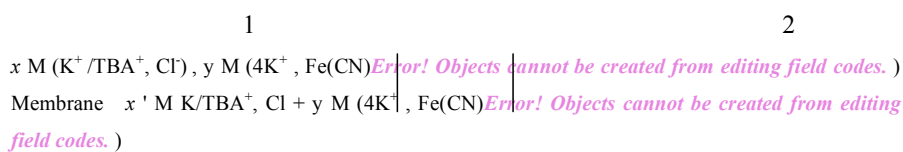
$$= I_F + \frac{dQ}{dt} = I_F + I_C \quad (3)$$

$$v_i = k_i^{1 \rightarrow 2} \left((c_{i1} - c_{i2}) \exp \left[-z_i F \left(\Delta\phi_1 - \Delta\phi_2 \right) / RT \right] \right) \quad (4)$$

Where A is the effective area of the membrane, ϕ_1 and ϕ_2 are the electrochemical potentials in phase 1 and 2 and c_{i1} and c_{i2} are the concentrations of permeable ions in phase 1 and 2 respectively and $k_i^{1 \rightarrow 2}$ is the potential dependent rate constant of ion transfer from phase 1 to phase 2 across the membrane.

As the capacitance of the membrane is fixed, the ionic current flows until the charge on the capacitor is satisfied. An excess of electrolyte solution satisfies this condition. As increasing potential gradient is applied, an increasingly polar environment is created within the ion channels. In contact with ions of appropriate radii and charge, an increase in current is expected as ions pass through the channels.^{23,24}

In our experiments, an egg phosphatidylcholine bilayer lipid membrane (BLM) formed over a microporous polycarbonate membrane support²³ was inserted between two aqueous electrolyte solutions containing equal and excess concentrations of the redox active Fe(CN)₄⁻ ion to maintaining an equal potential on either side of the membrane and allowing the electrode to remain unpolarisable for ionic current measurements (for scenario 2 and 3 *vide infra*). The schematic configuration is:



With the BLM inserted between two aqueous solutions three different scenarios exist:

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1) The control membrane comprised of a bilayer lipid membrane (BLM) painted on a polycarbonate membrane behaves as an insulator, except when exposed to potentials high enough to porate the membrane (E_{porate}). In presence of an electroactive species, Fe(CN) $\text{Error! Objects cannot be created from editing field codes.}$, therefore, if the UME tip potential is held at a value conducive to reducing the Fe(CN) $\text{Error! Objects cannot be created from editing field codes.}$ species, as the tip approached the insulating surface, tip current is affected by hindrance of the diffusion of the electroactive species, showing a negative feedback approach curve.

2) With a gA inserted in the lipid membrane, the ion channel forms a locus at which permeable cations can cross the membrane and accumulate depending on the concentration gradient. Approach curves measuring ionic current (see experimental section) show pH and temperature dependent conducting behaviour, which is **independent** of the potential gradient applied to the membrane.

3) With a gAN inserted membrane, the channel is closed when gAN is oxidised and opened when it is reduced (*vide infra*). Ions of suitable size and charge are able to flow through the channel when it opens. Approach curves therefore show a potential **dependent** behaviour.

The exact theoretical treatment of IT and ET processes in this situation is complex and will need further investigations to obtain a complete theoretical analysis.

Area scans in ferricyanide solutions were performed to monitor the flatness of the membrane and any detect any pin-hole defects. Cliffel and co-workers²⁵ report the observation of the voltage dependent opening and closing of alamethicin pore bundles in phosphatidylcholine BLMs using the redox active Ru(III) hexamine cation as transporting cation using an SECM UME with RG (ratio of the insulator radius to the disk electrode radius) values of $1.4(\pm 0.1)$. In the case of a small peptidic channel such as gA, which has a pore radius of ~ 0.2 nm in the $\beta^{6,3}$ conformation, we do not expect to observe single channels; however the behaviour of channel porated membranes resulting from ion permeation through clusters of channels which form a locus for ion accumulation, may be observable. Literature precedents suggest that at a concentration of 2 mol% gA forms clusters of approximately 50-100 gA dimers in phospholipid membranes.¹⁹ The lateral resolution in our experiments was insufficient to enable this observation.

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Experimental:

Chemicals

Gramicidin D (Sigma, UK) containing ~80 % gA was vacuum dried over phosphorus pentoxide for 24 hours before use. Ethylenediamine, 4-nitrophenylchloroformate, 2,4-dinitrochlorobenzene and nicotinamide (Aldrich, UK) were used as purchased. All solvents were of HPLC grade (Fisher Scientific, UK) and were twice distilled and purged with nitrogen before use. All other reagents were of electrochemical grade and were used without further purification.

Equipment

SECM data were collected using a Uniscan Instruments SECM 270 (Uniscan Instruments Ltd, Buxton, UK). This apparatus comprised a precise tip-positioning device, a bipotentiostat and associated software. A two-compartment glass cell, with inter-compartment aperture, was used. The SECM was used in two modes. For monitoring the insulating behaviour and area curves for a BLM modified membrane, the ultra microelectrode (UME) was a 10 μm platinum SECM tip (UME) (CH instruments Ltd, Austin US) with an aqueous Ag/AgCl reference electrode (RE) (BAS, West Lafayette, US) and a 1 cm^2 platinum flag counter electrode (CE). To monitor ionic currents within the voltage window for Fe(CN) $_6^{3-}$ reduction (121 mV vs Ag/AgCl), for ion channel impregnated membranes, the SECM UME acted as a counter electrode with the Ag/AgCl reference. A second Pt WE was placed in phase 2 and connected to the bipotentiostat was used to measure potential gradients across the membrane. A constant excess $\text{K}_3\text{Fe}(\text{CN})_6$ solution on either side of the membrane ensured a constant redox potential. Data was collected by a personal computer running the SECM 270 software supplied with the microscope. Lipid bilayers were prepared on Whatman Track-Etched polycarbonate membranes, of 25 mm diameter and 100 nm pore size. For cyclic voltammetry (CV) and chronoamperometry (CA) measurements the tip potential and sweep rates were controlled by a PG580 potentiostat (Uniscan Instruments Ltd, Buxton, UK) using the SECM 270 software in either CV or CA

mode. The three electrodes were placed in electrolyte solution in a 10 ml cylindrical glass cell. Measurements were made in stationary solution, with stirring between experimental runs.

Reverse-phase high-pressure liquid chromatography (RP-HPLC) was carried out at 25 °C using a C8 semi-preparative column (25 cm × 10 mm) of 5 µm particle size (Supelcosil LC-8). gAN was applied to the column as a methanolic solution. A ternary mobile phase was used for gAN elution: solvent A: acetonitrile; solvent B: isopropanol/water (1:19); solvent C: methanol/water (9:1). All solvents contained 0.1% TFA by volume. The following gradient was used:

%A	%B	%C	Time (min)	Flow rate (ml/min)
0	100	0	1	2
2	28	70	2	2
2	0	98	2	2
5	0	95	10	2
10	0	90	3	2
0	100	0	1	2

gAN elution was monitored at detector a wavelength of 280 nm.

Electrospray ionisation mass spectrometry (ESI-MS) was performed with a Micromass LCT spectrometer.

Synthesis of gram-2-(nicotinamido)ethyl carbamate (gAN)

Gramicidin-ethylenediamine (gE) was synthesised by Woolley's method.²⁶ 2,4-dinitrophenyl-pyridinium-3-carboxamide chloride (DNPC) was synthesised according to the method of Lettré.²⁷

Crude gE (210 mg, 0.11 mmol) was dissolved in methanol (1.5 ml). DNPC (38 mg, 0.12 mmol) was added *portionwise* to the *stirred* solution, *resulting* in an *immediate* colour change from pale yellow to dark red. The mixture was stirred at room temperature for *a further* 2 hours, *before the* addition of ice-cold high purity water (10 ml), *resulting* in the *formation of a* pale yellow *precipitate*. *Following centrifugation* at 3000 rpm for 3 minutes, *the supernatant was removed by decantation*. *After triturating* the pellet *three times* with 10 ml of *ice* cold water, *with removal of the*

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solvent each time by centrifugation/decantation, the product was dried for 24 hours over phosphorus pentoxide. The crude product was purified by RP-HPLC to yield the title compound (162 mg). ESI-MS: $m/z = 2074.4$ [M^+] (calc = 2074.5). HPLC retention time = 14.8 min. The HPLC chromatogram of the purified gAN is shown in Figure 2.

Sample preparation

Egg phosphatidylcholine (EPC) in chloroform (100 mg/ml; 200 μ l) was transferred to a 50 ml round-bottomed flask and the solvent removed *in vacuo*. The dry EPC residue was then suspended in high purity water (1 ml) and the mixture agitated using a vortex mixer for 15 minutes. The lipid mixture was then painted onto polycarbonate membranes, which were resting on a non-absorbent plastic surface. Following air-drying, the membranes were turned over, painted on the reverse and allowed to air dry again. To compare the behaviour of the gAN/gAN⁺ couple in KCl/(CH₃)₄NCl supporting electrolytes the following solutions were prepared in sample bottles: (a) 2 $\times$ solutions of 100 μ l of methanol and 900 μ l of high purity water, (b) 2 $\times$ solutions of 100 μ l of 0.12 mM gAN in methanol and 900 μ l of high purity water (concentration of gAN = 1.2×10^{-5} M), (c) 2 $\times$ solutions of 100 μ l of 0.12 mM gA in methanol and 900 μ l of high purity water (concentration of gA 1.2×10^{-5} M). To determine the pH dependence of the gAN/gAN⁺ couple an additional 7 $\times$ solution (a) were prepared.

An EPC painted membrane was placed into each sample bottle and refrigerated for 24 hours prior to use.

Chronoamperometric measurements:

A polycarbonate membrane was secured over a 1.6 mm diameter platinum electrode (Bioanalytical Systems, US) with a rubber o-ring. The membrane was positioned so that the polycarbonate was tight to the underlying platinum surface. EPC was painted onto the polycarbonate surface. During EPC application the polycarbonate was smoothed with the brush to prevent air bubbles being trapped between the platinum and membrane. The EPC was left to air dry before the electrode was suspended in a solution of 100 μ l of 0.12 mM gAN in methanol and 900 μ l of high purity water (concentration of gAN = 1.2×10^{-5} M) and refrigerated overnight. The electrode was then immersed in a 100 mM tris buffer (pH 7.0)/1 mM K₃Fe(CN)₆ solution and CA

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performed with a sequence of 10 s potential-steps between -0.55 V ($t = 0$ s) and -0.1 V. Measurements were repeated with TiNO_3 and $\text{Eu}(\text{NO}_3)_3$.

SECM measurements

The Pt electrodes were thoroughly cleaned by soaking in a 3:1 (v/v) mixture of concentrated sulphuric and nitric acids for 2 minutes, followed by sonication in high purity water for 2 minutes. The electrodes were then rinsed with methanol and allowed to air dry. The radius of the tip, a , was measured as 10.3 μm using the diffusion limiting current.

Figure 3 shows a schematic diagram of the cell used. Both compartments of the cell were filled with equimolar concentrations of 0.1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ solution and a membrane was carefully mounted on the inter-compartment aperture. The open circuit potential at either side of the membrane was monitored and was 131 mV vs Ag/AgCl. The Pt UME (WE1) in phase 1 was used both as an SECM WE (with CE1 as counter electrode) and as CE in ion-amperometric mode. The cell was clamped in the SECM cell inside a Faraday cage and adjusted to horizontal with a spirit level. The flatness of substrate was checked by area scans and feedback currents (area scans were performed on an EPC painted polycarbonate using a Pt UME was positioned in close proximity to the lipid surface and at a potential of 0.1 V vs Ag/AgCl. The tip was rastered across a $50 \times 50 \mu\text{m}$ section of the membrane at a rate of $1 \mu\text{ms}^{-1}$). The platinum UME tip was positioned approximately 10 μm perpendicular to the membrane plane. The tip-substrate distance was monitored by a video camera and controlled by carefully moving the tip towards the substrate to obtain maximum current. On occasions where the tip accidentally touched the membrane, a dip in current was observed. Approach curves were obtained as a function of distance, d , by moving the tip away from the surface at a speed of $1 \mu\text{ms}^{-1}$ and normalizing the tip current by the diffusion limiting current. The concentrations of monovalent ions were varied between the two compartments. The concentration in phase 1 was either 2 mmol dm^{-3} or 100 mmol dm^{-3} and the concentration in phase 2 was reversed depending on whether the ingress or egress of ions was monitored.

pH dependent experiments

Potassium hydrogen phosphate buffers with pH 6.5, 7.0, 7.5 and 8.0 by using the appropriate ratios of the mono and dihydrogen phosphate salts were used to change

the pH in both phases. The concentration ratios of potassium ions in both phases were maintained.

Temperature Dependent Studies:

The two-compartment double walled SECM cell was filled with pH 7.0 phosphate buffer and 0.1 mM $\text{K}_3\text{Fe}(\text{CN})_6$. The electrolyte temperature was regulated by passing heated water through the cell jacket. A thermostatic bath (Fisons Haake D8, accuracy ± 0.2 °C) was used and the electrolyte temperature was double-checked by digital thermometer. Approach curves were obtained at electrolyte temperature of 0, 10, 20, 30, 40 and 50 °C.

Results and Discussions:

The half wave potential of gAN at pH 7 and 298 K was measured at -500 mV vs. a saturated calomel electrode (SCE) (compared to -560 mV for the NAD^+/NADH couple).²⁸ The half wave potential for aqueous solutions for the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ couple is approximately 121 mV vs SCE. At potentials lower than the redox potential of gAN the ion-channel should open. The limit of the potential window is defined by the half wave potential of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ couple and is adequate for our measurements.

Chronoamperometric experiments at which the electrode potential was switched between channel opening (-0.55 V) and channel closing (-0.1 V) potentials showed reversible switching behaviour (Fig 4) in presence of K^+ and Tl^+ , but not in presence of Eu^{3+} ions. Gramicidin channels are ideally permeable for monovalent alkali metal cations. Higher valence cations like Eu^{3+} block the channel by binding near the mouth.²⁹ K^+ and Tl^+ have similar charge and size (K^+ : Ionic radius = 152 pm (6 co-ordinate, octahedral), Tl^+ : Ionic radius = 164 pm (6 co-ordinate, octahedral), Eu^{3+} : Ionic radius = 108.7 pm (6 co-ordinate, octahedral)).³⁰ The equilibrium binding constant for K^+ and Tl^+ have been reported as 52.6 mol^{-1} and 582 mol^{-1} respectively.³¹ The chronomperograms reflect this trend, showing a higher current and more facile influx of Tl^+ compared to K^+ . Eu^{3+} appears to block the channel.

The control membrane shows negative feedback up to -0.6 V indicating the insulating nature of the membrane. At an applied potential of -0.8 V an increase in current is observed which is, most likely, indicative of membrane rupture.

gA inserts into BLMs to form a head-to-head dimer composed of right-handed β^3 helices that span the bilayer and form cation active channels. The hydrophobic side chains of amino acids such as valine and leucine are directed outwards towards the hydrophobic lipid bilayer and the polar carbonyl groups orient towards the lumen of the channel, resulting in a polar environment within the channel to aid the passage of monovalent cations. gA channels are normally active until the dimer dissociates into two monomers. The gA porated membrane shows distinctly different behaviour in the presence of ion-channel permeable K^+ and the larger $(CH_3)_4N^+$ ion (Fig 5b). With K^+ positive feedback currents at potentials between 0.1 V and -0.6 V indicate an influx of ions. A profile of the tip current at membrane (at $x \rightarrow 0$, but not touching the membrane, Fig 6) shows potential independent behaviour within experimental error. This observation agrees well with previous reports of the approximate electroporation threshold for gA of 0.3 V.³² In presence of $(CH_3)_4N^+$, a negative feedback current at -0.5 V (Fig 5b) indicates a suppression of ion flux. This work is presented to emphasise the redox potential modulated behaviour of gAN (*vide infra*) in comparison to gA.

gAN *is expected to* insert in the lipid layer with the hydrophilic nicotinamide moiety facing the aqueous phase on each side of the lipid painted membrane. With the gAN inserted membrane, it is observed that at a tip potential of approximately -0.52 V the ionic current in K^+ concentration gradients shows a sudden increase. We believe this is the potential at which the nicotinamide moiety is fully reduced allowing the channels to open. This observation is supported by chronoamperometric experiments (*vide supra*). Once again, in concentration gradients with the larger $(CH_3)_4N^+$ ion, an ingress of ions is not observed as shown in Fig 5c. The ionic current profile at $x \rightarrow 0$, (Fig 6) for gAN, clearly pin points the voltage gating potential at -0.51 V.

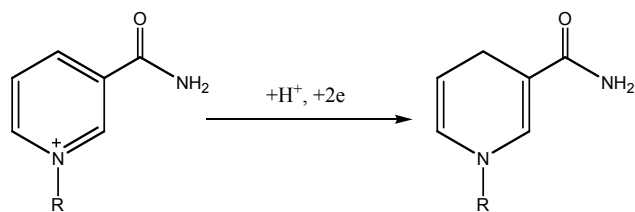
The pH dependent effect of the reduction of the nicotinamide moiety in gAN and the corresponding shift in the voltage gated channel opening, which becomes more facile and shifts to lower potentials with decreasing pH, is to be expected and is clearly evident (Fig 7). The switching potential changes from -0.7 V at pH 8 to -0.13 V at pH 6.

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The voltage switch appears to be independent of temperature at the range of temperatures studied (0-50 °C)(Fig 8).

Conclusions:

This study confirms the voltage gating properties of a newly synthesised gramicidin analogue using a combination of chronoamperometry and SECM in ion amperometric mode. The switching potential at which the nicotinamide moiety is reduced thereby opening the ion channel is pH dependent, with a value of -0.5 ($\pm$ 0.05) mV at neutral pH. This type of ion-channel regulation is very interesting for a range of applications such as ion-selective sensors, drug-delivery, mechanistic studies on charge and voltage control of ion channels amongst others.

Key to figures:

- 1) gAN is formed by the C-terminal addition of the nicotinamide moiety (shown in red) to gA (shown in black).
- 2) HPLC trace of purified gAN.
- 3) SECM cell diagram.
- 4) Chronoamperometry of gAN membrane with different permeant cations. Inset shows pulse pattern applied.
- 5a) Approach curves for control membrane (untreated with peptide), showing insulating behaviour at -0.6 V and electroporation at -0.8 V. The broken lines show the approach curves for an ideal insulator (I) and conductor (C).
- 5b) Approach curves for a gA porated membrane, showing *voltage independent* permeation of K⁺ and channel blocking properties of (CH₃)₃N⁺ ions.
- 5c) Approach curves for a gAN porated membrane showing *voltage dependent* permeation of K⁺. Insulating behaviour is observed up to -0.51 V beyond the potential an increase in ionic current is observed corresponding to the switching potential observed with the CA experiments.
- 6) Comparison of gA and gAN porated membrane ionic currents at (x @ 0), showing the switching potential of the gAN porated BLM.
- 7) Dependence of gAN switching potential on pH.
- 8) Dependence of gAN switching potential on temperature.

Fig 1.

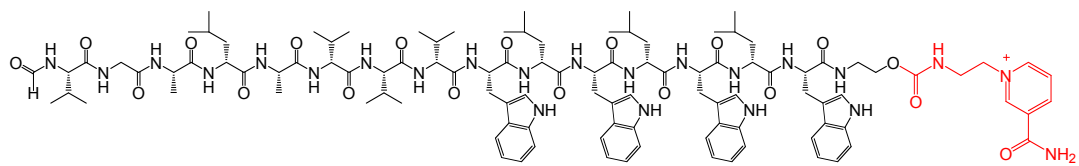


Fig 2.

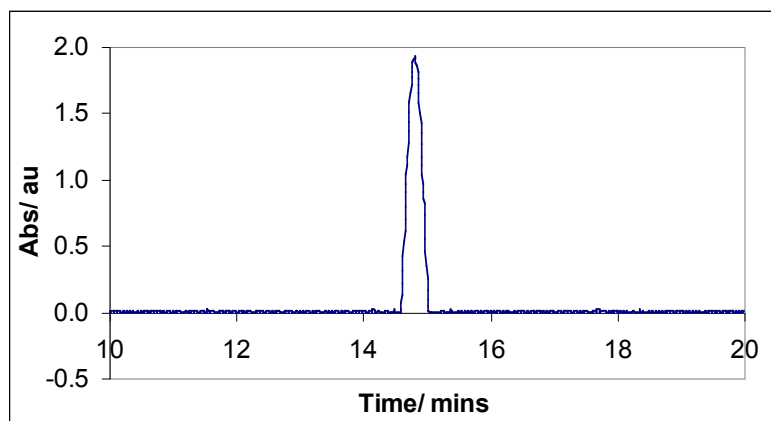


Fig 3.

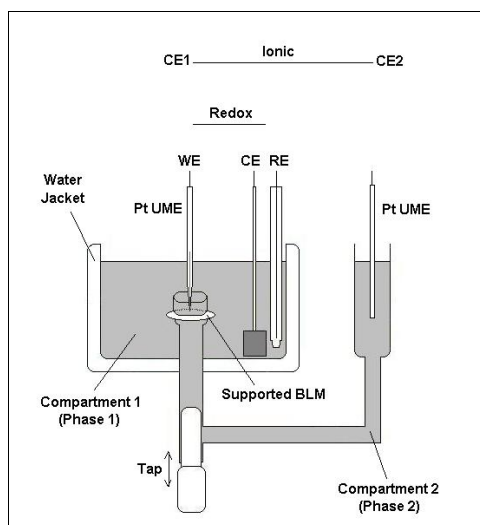


Fig 4.

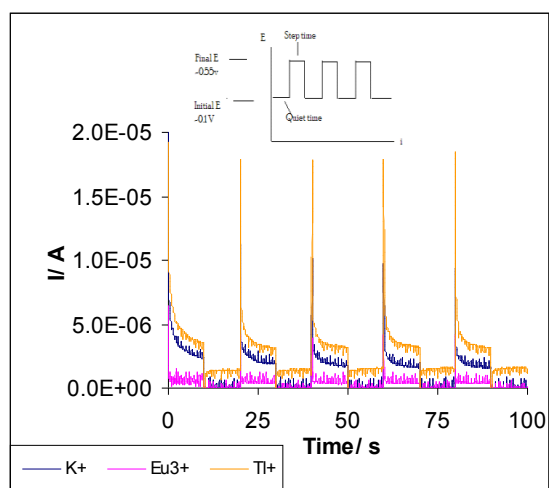


Fig 5a.

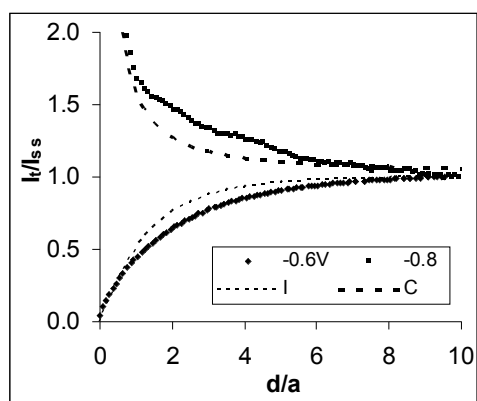


Fig 5b.

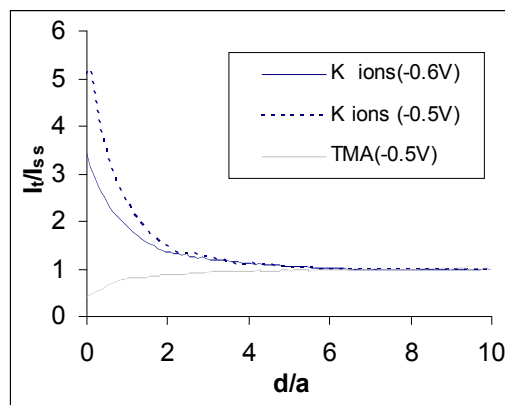


Fig 5c.

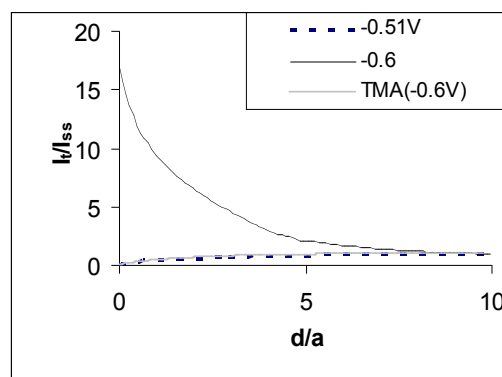


Fig 6.

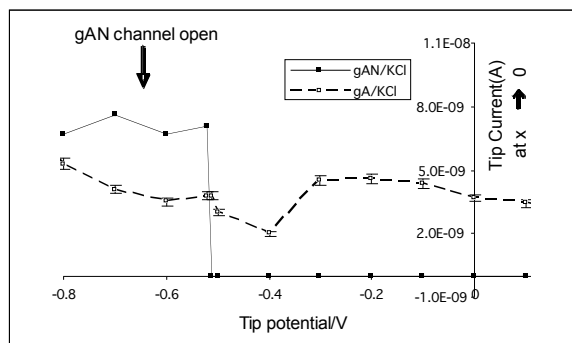


Fig 7.

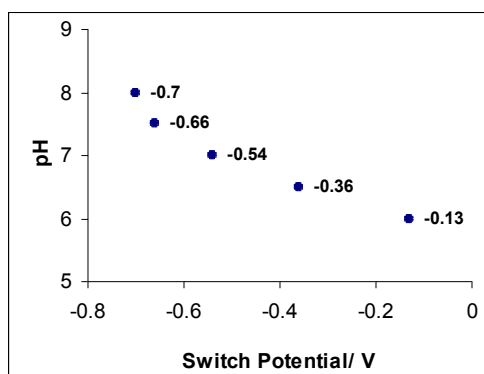
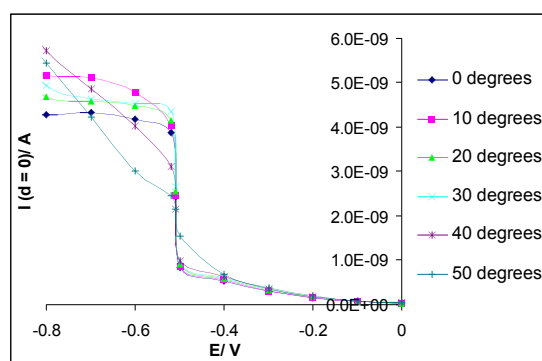


Fig 8.



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