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Membrane activity of biotechnological peptide drugs

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Charged Langmuir–Blodgett monolayers deposited at an immobilised liquid–liquid interface have been used as a simple model for a biological membrane to investigate the membrane activity of biotechnological oligopeptide drugs.

The membrane activity of peptides is determined by a sensitive balance of hydrophobic and electrostatic interactions.[1] Accordingly, charge optimisation was found to increase the antimicrobial activity of magainin peptides, an effect directly caused by the interaction of the peptides with the lipid matrix of the target membrane.[2] In another study, the delivery of somatostatin-related peptides to their membrane-bound receptor was found to depend on both the hydrophobicity and the electrostatically induced surface concentrations of the peptides.[3]

The use of electrified liquid–liquid interfaces for investigating ionic drug transfer has undergone rapid development in the past decade.[4] In this study, we employ electrochemistry at monolayer-modified liquid–liquid interfaces to investigate the membrane activity, that is, the surface concentration of the drug, of two structurally related decapeptides of pharmaceutical importance, LHRH (leutinising hormone releasing hormone) and nafarelin (synthetic analogue of the gonadotropin-releasing hormone (GnRH)).† The peptides are electrically similar and their chemical structure differs by just one amino acid, glycine in LHRH is replaced by the more hydrophobic alanine in nafarelin, but studies have suggested that this is sufficient to induce a difference in their membrane activity.[5] The membrane activity of the peptides is studied as a function of peptide and membrane charge employing ac voltammetry at a biomimetic liquid–liquid interface. To this end a lipid monolayer was deposited at a water–nitrophenyl octyl ether (NPOE) gel interface using the Langmuir–Blodgett technique. The electrochemical setup and deposition procedure have been thoroughly described in previous papers.‡[6] In this study, the monolayer was formed with distearoyl phosphatidylcholine (DSPC) mixed with stearic acid (STA).

The monolayer-modified liquid–liquid interface contains certain advantages over lipid covered metal electrodes in that i) the substrate is a gellified liquid, which does not constrain the layer; ii) the setup enables ion transfer to be observed, which is not measurable at solid supports. Thus, simultaneous information on membrane activity and phase transfer is attainable.

The peptide–monolayer interaction was assessed based on the interfacial capacitance data derived from the ac voltammetry measurements. Capacitance is a sensitive indicator of the dielectric properties and charge of the liquid–liquid interface. Furthermore, models based on the solution of the Poisson–Boltzmann equation allow interfacial electrostatic parameters to be obtained in a semi-quantitative manner. The model used in this communication has been described in detail previously.[7] Briefly, the theory involves an ion-free layer with a certain surface charge density (σ) and dielectric permittivity to thickness ratio (ε_M/d) sandwiched between two electrical double layers that are characterised by the Debye lengths of the organic ($1/\kappa_o$) and aqueous ($1/\kappa_w$) phases, see Fig. 1. Based on the numerical solution of the problem,[7] the shift in the minimum of the capacitance *vs.* interfacial Galvani potential difference, $\Delta_o^w\phi$, is practically independent of ε_M/d, *i.e.* only a function of the surface charge density. In the limit of large ε_M/d, a relation between the surface charge and the potential of the capacitance minimum, $\Delta_o^w\phi_{pcm}$, can be obtained as follows (based on the theory presented in reference 7):

$$\Delta_o^w\phi_{pcm} = 2RT/F \ln\left(-\alpha + \left(\alpha^2+1\right)^{1/2}\right) \quad (1)$$

where $\alpha = F^2/(2RT)\Gamma_{charge}/(\varepsilon_w\kappa_w)$ and Γ_{charge} is the total charge of the monolayer (charge of the monolayer + the charge of the adsorbed molecules in units of surface concentration, $\sigma = F\Gamma_{charge}$). Comparison with the numerical solution showed that eqn. (1) is accurate to within 5 mV for typical values of parameters (Γ_{charge}, Debye lengths and ε_M/d).

Peptide–membrane interactions could not be observed at an uncharged membrane (a pure DSPC monolayer) at either pH 6 or 9. If the peptides would adsorb on the monolayer, the charge they carry should induce a shift in the capacitance *vs.* potential curve. Thus, hydrophobic interactions between phosphatidylcholine monolayers and the peptides under study are either non-existent or too small to be measured by this technique. In addition, the peptides are too hydrophilic (the Gibbs energy of transfer between the aqueous and organic phases is too large) to be transferred through the liquid–liquid interface in response to the potential difference applied.

Both peptides appear to interact with a charged membrane, a mixed monolayer of STA/DSPC (45/55 mol%) at pH 6.2. Fig. 2 shows the interfacial capacitance as a function of the Galvani potential difference. In each of the figures, trace A (dotted line) has been recorded in the absence of the drug (pH 6.2) and the curves B (solid line, pH 6.2) and C (dashed line, pH > 9) in the presence of 0.2 mM of either LHRH (Fig. 2a) or nafarelin (Fig. 2b). Whereas LHRH seems to be membrane active only at pH 6.2, nafarelin adsorbs at both pHs. In order to quantify these observations, the theory described previously is used to extract the surface charge in each of the experiments.

The monolayer was deposited at a mean molecular area of 40 Å^2, which corresponds to the surface charge concentration of -1.85×10^{-6} mol m^{-2}. The stearic acid is fully deprotonated at the pHs used in this study (pK = 4.7[8]) and due to buffering, the interfacial pH is not sensitive to potential. The difference between the value obtained for this surface charge from theory (0.175 V) and the measured value of *ca.* −0.02 V can be attributed to a dipole potential due to the dipoles of the monolayer forming materials.[6,9] It is further assumed here that this dipole potential is constant, *i.e.* not a function of the pH or

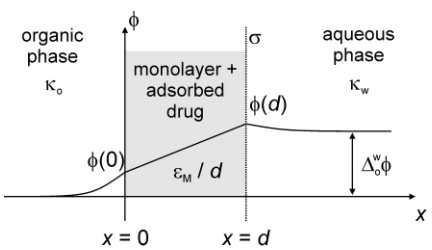

Fig. 1 A schematic of the theoretical model.

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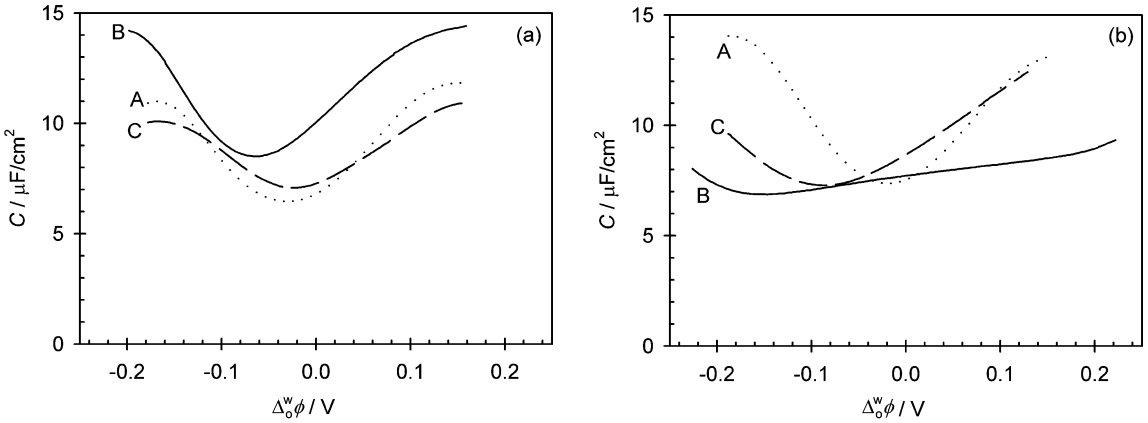

Fig. 2 Interfacial capacitance of a mixed STA/DSPC monolayer-modified interface (A) and in the presence of (a) 0.2 mM LHRH (pH 6.2 (B) and 9.8 (C)) and (b) 0.2 mM nafarelin (pH 6.2 (B) and 9.3 (C)).

the amount of adsorbed peptide. Using this type of approach, the surface charge concentration in the presence of LHRH at pH 6.2 is obtained as -1.0×10^{-6} mol m^{-2}, indicating that roughly half of the membrane charge has been shielded by the peptide. However, in the case of nafarelin at pH 6.2, this residual charge is only -0.1×10^{-6} mol m^{-2}, suggesting that over 90% of the initial charge has been screened by the peptide.

The effect of increasing the pH is two-fold: it affects the peptide charge as well as the surface concentration. In the case of LHRH, the original surface charge is restored upon increasing the pH, *i.e.* the peptide is no longer membrane active. In the case of nafarelin, however, there is a significant shift in the capacitance curve even at the higher pH. Analysis yields a surface charge of -0.5×10^{-6} mol m^{-2}. However, if the change in peptide charge (*ca.* +1.4 at pH 6.2 and *ca.* +0.9 at pH 9.3, calculated based on the p*K* values of the amino acids[10]) is taken into account, actual surface concentrations of nafarelin are 1.2×10^{-6} mol m^{-2} (pH 6.2) and 1.5×10^{-6} mol m^{-2} (pH 9.3). The actual surface concentration for LHRH is low, *ca.* 0.3×10^{-6} mol m^{-2} at pH 6.2, and ≈ 0 at pH > 9.

The shape of the capacitance curves gives further insight into the structure of the adsorbed peptide layers as it is sensitive to ε_M/d. With the exception of nafarelin at pH 6.2, the curvatures do not change significantly; this suggests that the presence of the adsorbed layer does not strongly affect the dielectric properties of the interfacial region. That is, the adsorbed layer is thin and/or non-compact. However, with nafarelin at low pH, the capacitance curve is considerably flattened. The model gives an estimate of $\varepsilon_M/d \approx 0.2$, which is less than half of the value obtained for the other cases. This could indicate the formation of a compact peptide–lipid layer at the interface. Such behaviour could be rationalised in terms of the peptide charge; the more highly charged peptide might preferentially adsorb in a flat conformation in order to provide access for all charged groups to the interface.

The difference in the membrane activity between the two peptides is likely to result from nafarelin being more hydrophobic than LHRH. Thus upon association with the membrane surface nafarelin can be expected to penetrate deeper into the monolayer, forming a more compact layer at the interface than LHRH. Similar trends have been observed in a previous study, although in this case LHRH was not found to exhibit any membrane activity at all.[5] According to the results obtained with nafarelin, both the lipophilic residue and the adjacent positive charge in the molecule were hypothesised to be crucial to the drug adsorption in the skin.[11]

Despite carrying a more hydrophobic substituent group, nafarelin does not appear to be membrane active at a neutral monolayer. This is likely to result from the overall very hydrophilic nature of the peptides. As a consequence, to observe detectable membrane activity, electrostatic peptide–membrane interactions are required to increase the surface concentration of the peptide, thereby facilitating its association with the membrane. Such an observation is important, since most biological membranes carry an overall negative charge, the charge density of which depends on the membrane.

This study demonstrates that electrochemistry at a monolayer-modified liquid–liquid interface is a versatile and sensitive tool to study the membrane activity of biological compounds, yielding information on the surface concentration and conformational changes of the adsorbing species. In addition, the methodology allows for possible simultaneous charge transfer reactions across the interface to be studied. The results obtained in this study support previous findings that peptide–membrane interactions are altered by fine changes in charge and/or hydrophobicity. Accordingly, nafarelin was found to be significantly more membrane active than LHRH, while the membrane adsorption of both peptides was strongly affected by modest changes in their charge. A detailed study of the effects of concentration and interfacial potential difference on the adsorption behaviour will be the subject of further studies.

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Notes and references

† LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was purchased from Sigma and nafarelin (pGlu-His-Trp-Ser-Tyr-Ala-Leu-Arg-Pro-Gly-NH_2) was synthesised at the Department of Biochemistry in the University of Kuopio (Kuopio, Finland).

‡ The organic phase was immobilised by addition of 5 wt% of PVC (very high molecular weight). The organic base electrolyte was 10 mM bis(triphenylphosphoranylidene)ammonium tetrakis(4-chlorophenyl)borate and the aqueous phase contained 10 mM LiCl; pH was buffered to the value of 6.2. The pH was adjusted to > 9 by addition of LiOH.

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