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Peptide−Phospholipid Complex Formation at Liquid−Liquid Interfaces

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Two peptides known to interact with receptors embedded in cell membranes, angiotensin III (AngIII) and Leu-enkephalin (LeuEnk), were studied electrochemically at the interface formed between two immiscible electrolyte solutions modified by an adsorbed monolayer of dipalmitoylphosphatidylcholine (DPPC). The results indicate that cationic angiotensin III transfer can be facilitated by the interfacial formation of a complex with DPPC. The complexation constant was determined by voltammetry and found to be equal to 5.2×10^4 M^{-1}. For neutral Leu-enkephalin, a current only observable in the presence of the lipidic monolayer results from the formation of a complex between the lithium cation, LeuEnk or LeuEnk dimer and the phospholipid. For both peptides, the peptide−lipid complexes were identified by biphasic electrospray ionization mass spectrometry using a setup consisting of a dual-channel microchip, which puts in contact two immiscible phases at the Taylor cone and makes possible the study of interfacial complexes. The stability of the 1:1 complexes between lysine, diphenylalanine, AngIII, and LeuEnk and DPPC were evaluated by varying the temperature of the heated capillary of the mass spectrometer. Finally, from the complementary use of voltammetry and mass spectrometry, a mechanism for the interaction between these two biologically relevant peptides and DPPC monolayers is formulated.

Phospholipid monolayers adsorbed at the liquid−liquid interface formed between two immiscible electrolyte solutions (ITIES) have been widely used to mimic the behavior of the lipid biomembranes,[1] especially to investigate the stability[2−4] and ion permeability[5−7] of the monolayers as a function of the well-defined interfacial potential difference.

On the other hand, amino acids, oligopeptides, and analogues have also been studied using liquid−liquid electrochemistry[8] with the aim of determining their lipophilicities and their likelihood to transfer through biological membranes. Thus, in order to obtain insight into the interaction between peptides and phospholipid monolayers, we have investigated electrochemically the interaction between L-α-dipalmitoylphosphatidylcholine (DPPC), a zwitterionic major constituent of cellular membranes,[9] and two different peptides, namely, angiotensin III (AngIII) and Leu-enkephalin (LeuEnk), at the water−1,2-dichloroethane (DCE) interface. These two peptides are known to interact with receptors embedded in the cellular membrane. For example, AngIII stimulates receptors of the rennin−angiotensin system that is known to play an important role in the regulation of blood pressure,[10] and Leu-enkephalins also play an important role in the inflammatory and immune response by acting as neurotransmitters that could interact with the membrane lipids prior to receptor binding.[11] This clearly suggests that the membrane acts not only as an inert matrix for the receptors but also as a catalyst for the peptide−receptor interaction.[12]

However, the membrane-forming lipids are also known to interact with different compounds such as proteins, peptides, and metallic cations. Such a variety of compounds and interactions require different analytical approaches to probe the lipids themselves and their interaction partners. Imaging, when using living cells, or nuclear magnetic resonance (^{1}H in general and ^{31}P for phospholipids), chromatography (either thin-layer chromatography or liquid chromatography), mass spectrometry (MS), and biochemical assays, when using extracts, are the methods of choice.[13,14] Among these techniques, MS has become an important tool thanks to the advances in ionization techniques and its wide range of use. Usually, for the MS analysis of lipids, they are extracted with an organic solvent mixture and analyzed by electrospray ionization (ESI) techniques in chloroform/methanol mixtures.[15−19] ESI sources are suitable for studying biomolecules

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Scheme 1. Electrochemical Cell Employed

Ag | AgCl	LiCl 10 mM BTPPACl 1 mM	10 mM BTPPABTPFB + x μM DPPC	10 mM LiCl (pH ≈ 5) + y μM AngIII / LeuEnk	AgCl | Ag

and noncovalent binding due to its softness,[20−22] and great efforts have been made for its development. Cooling of the source, as performed by Robinson and co-workers to study multimeric proteins complexes[23] or by Yamaguchi with the cold-spray ionization;[24] fabrication of various emitters and multicapillary inlet to improve MS sensitivity;[25−27] electrochemical cells coupled to ESI;[28] and microfabricated electrospray devices[29,30] are only few examples. Another approach consisting of a two-phase microflow system was also proposed by Watarai and co-workers where an organic solvent is injected in an aqueous sheath solution.[31,32]

In particular, our laboratory has developed polymer microsprayers with built-in electrodes for tagging of peptides,[33] studying bioinorganic complexes, or both.[34−36] A dual-channel microsprayer has been also used to tag phosphopeptides.[37] Recently, such a microsprayer has been used to study a biphasic system in MS, where cations or peptides in water and thioether crowns or ether crowns dissolved in DCE were put in contact within the Taylor cone[38] formed during the electrospray process.[39]

In the present paper, peptide−lipid interactions were monitored by cyclic voltammetry in order to investigate the mechanisms of the interfacial transfers that could take place for both systems. The complementary technique of biphasic electrospray ionization mass spectrometry (BESI-MS) was used to determine the stoichiometries of the complexes as well as to identify different ions that could shed some light on the association and desorption mechanisms that take place when both peptides and phospholipids are present at the water−DCE interface. Additionally, the stability of the complexes formed between DPPC and amino acids or peptides was studied and analyzed as a function of the peptide structure and the MS capillary temperature.

EXPERIMENTAL SECTION

Chemicals. Lysine (Lys, M = 146.2 g/mol) and DCE were bought from Fluka (Buchs, Switzerland). Diphenylalanine (FF, M = 312.4 g/mol), human AngIII (RVYIHPF, M = 931.1 g/mol), and LeuEnk (YGGFL, M = 555.6 g/mol) were from Bachem (Bubendorf, Switzerland), and the phospholipid DPPC (M = 734.0 g/mol) and acetic acid (AcOH) were from Sigma (St. Louis, MO). Methanol was from Riedel-de-Haën (Seelze, Germany), and deionized water (18.2 MΩ·cm) was prepared using a Milli-Q system from Millipore (Bedford, MA). For the electrochemical measurements, the organic salt used was bis(triphenylphosphoranylidene) ammonium tetrakis(pentafluorophenyl) borate ($BTPPA^+TPFB^-$). This salt was obtained by metathesis of bis(triphenylphosphoranylidene) ammonium chloride with lithium tetrakis(pentafluorophenyl) borate (all bought from Fluka). The organic-phase solvent used was DCE. All the other employed compounds were analytical grade and used as received, unless otherwise stated.

Electrochemical Measurements. Voltammetric measurements were carried out using a programmable potentiostat (Autolab-PGSTAT 30, Metrohm) and a glass cell. The cell was first cleaned by chrom-sulfuric acid and rinsed thoroughly with ultrapure water. The iR potential drop was compensated using a positive-feedback method. The electrochemical cell can be represented as shown in Scheme 1, where x = 0 or 10 μM; y values were varied and will be specified in the text. The double line in the scheme represents the water−DCE interface (area, 1.53 cm^2). All the potentials for ion transfer were referred to the half-wave potential of tetramethylammonium (TMA^+) obtained by adding at the end of each experiment a predetermined amount of tetramethylammonium chloride (TMA^+Cl^-) to the organic phase. The value of the standard transfer potential of TMA^+ has been taken equal to 0.16 V.[40] All the measurements were carried out at room temperature of 22 ± 1 °C.

Biphasic Electrospray and MS Setup. The biphasic electrosprayer was the one previously described.[38] Briefly, the microspray interface consists of a double microchannel (20 μm × 50 μm × 1 cm) polyimide microchip developed by DiagnoSwiss SA (Monthey, Switzerland).[37,41] The chip was fixed in a holder

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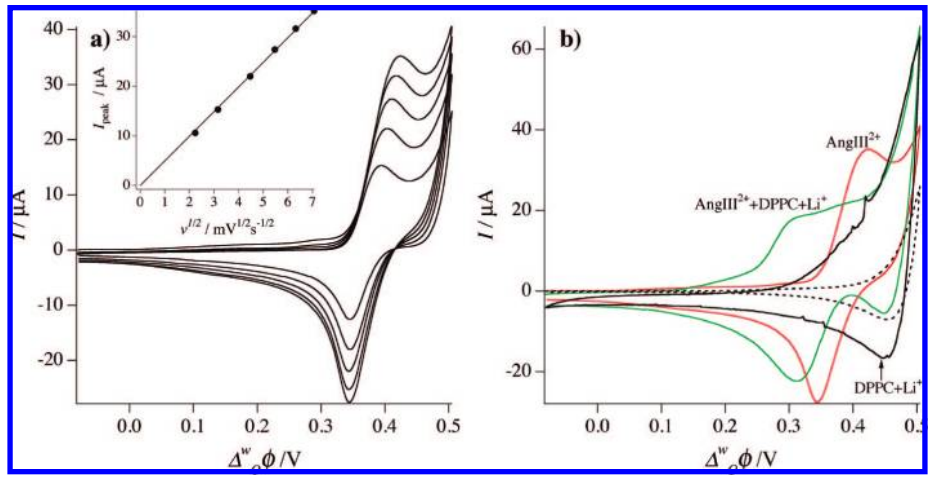

Figure 1. Ion-transfer voltammograms obtained at the interface formed between an aqueous solution of (a) AngIII ($y = 100$ μM) and DCE in the absence of DPPC at scan rates of 10, 20, 30, 40, and 50 mV·s^{-1}. (b) Voltammograms obtained for AngIII ($y = 100$ μM) in the absence (red line) and in the presence (green line) of DPPC ($x = 10$ μM). The voltammograms obtained for the supporting electrolyte in the absence (dashed line) and in the presence of DPPC (black line) are also presented at a scan rate of 50 mV·s^{-1}. The inset in (a) shows the linear relationship between the forward peak current and the square root of the scan rate.

connected to a syringe pump (KdScientific, Holliston, MA) able to hold two syringes (100 μL, Hamilton) and to introduce two different solutions (see Figure S-1, Supporting Information, SI). Two immiscible liquids are infused and put in contact only at the Taylor cone. In order to limit contaminations, one line was exclusively dedicated to the aqueous phase and the other to the organic phase.

Mass spectrometric measurements were carried out in an LCT time-of-flight mass spectrometer (Micromass, Manchester, UK) and in an LCQ DUO ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) both in positive ionization mode. The commercial electrospray interface was removed, and the microchip fixed onto a holder (as described above) was mounted on the probe slide adapter of the mass spectrometer. The high voltage was connected to the stainless steel needle of the syringe containing the aqueous solution. The pump was switched on (1 μL/min for each line, i.e., a total flow rate of 2 μL/min), and after the MS power supply onset ($U = 4.5-5.0$ kV), the chip was moved close to the entrance of the MS. The current was monitored by a nanoammeter. The mass spectra were averaged during 1 min. The complex stabilities were also evaluated by varying the temperature of the capillary at the entrance of the ion trap mass spectrometer.

RESULTS AND DISCUSSION

AngIII−DPPC System. Figure 1a shows the reversible ion-transfer voltammogram of AngIII present in the aqueous phase. The peak currents are proportional to the square root of the scan rate (inset Figure 1a). The peak separation was found to be 36 mV, close to the theoretical value for the transfer of a divalent ion, which evidently implies that under the experimental conditions AngIII carries two positive charges. This is in good agreement with the calculated charge of +2 in a pH interval comprised between 2.5 and 5, approximately (see Figure S-2a, SI). The half-wave transfer potential extrapolated to zero scan rate corresponds to 0.36 V, i.e., a Gibbs energy of transfer of 70 kJ·mol^{-1}.

On the other hand, when only DPPC is present in the cell (Figure 1b), a current increase can be observed at potentials above 0.3 V (black line) when compared with the blank (dashed line). This wave close to the end of the potential window has been observed previously by Yoshida et al.[42] and has been attributed to the formation of a complex between DPPC and the aqueous cation from the supporting electrolyte, DPPC acting then as an ionophore to facilitate the Li^+ transfer. This complexation process with Li^+ also produces structuring effects on monolayers due to its strong interaction with polar head groups of phospholipids as has already been reported.[43,44] However, when AngIII and DPPC are both present in the electrochemical cell, an additional wave appears before the peak for the diffusion-controlled transfer of AngIII^{2+}. This signal can be attributed either to the transfer of peptides adsorbed on the DPPC monolayer or to the transfer of aqueous peptides assisted by the phospholipid adsorbed at the interface. In the first case, we assume the presence of an adsorbed AngIII−DPPC interfacial complex that crosses the interface at potential less positive than the bare peptide, whereas a facilitated ion-transfer reaction corresponds to an interfacial complexation leading to the transfer of the aqueous ion. As shown in Scheme 2, both mechanisms result in a peptide−phospholipid complex in the organic phase, the main difference between the two scenarios being the assumption in the first case of an adsorbed peptide−phospholipid complex that transfers when the appropriate potential is applied. Indeed, the return peak in Figure 1b corresponds to the transfer of a complex cation more lipophilic than the bare peptide.

Figure 2 compares the voltammograms obtained for two different concentrations of AngIII. The current response present at ~0.375 V doubles when doubling the peptide concentration, whereas the current response at 0.295 V does not. Additionally,

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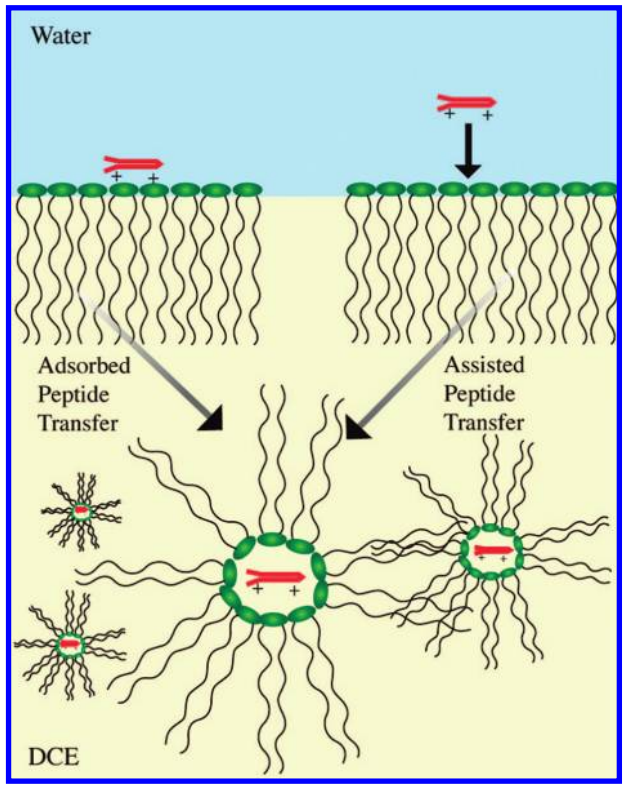
Scheme 2. Possible Mechanism for the Cationic Peptide AngIII Transfer Across the Water–DCE Interface Covered by a Monolayer of DPPC



the sweep rate dependence (Figure 2c) clearly shows that the current signal at 0.295 V is proportional to the scan rate, whereas that at 0.375 V is proportional to the square root of the scan rate (Figure 2d). This clearly indicates that the signal at 0.375 V is a diffusion-controlled ion-transfer reaction. The "noise" on the voltammograms is due to the mechanical instability of the ITIES in the presence of a strongly adsorbed monolayer strongly decreasing the interfacial tension. Nevertheless, the quality of the signal is sufficient to be conclusive.

The data presented are strong evidence for the adsorption of a complex between DPPC and $AngIII^{2+}$ that undergoes an electrochemical transfer reaction from the adsorbed state.[45] At the same time, it has to be noted that the adsorption of this complex must be strong, the latter due to the fact that prepeaks in cyclic voltammetry are basically the signature of a strong adsorption process. Weak adsorption generally implies that a separate peak cannot be observed and that the diffusion and adsorption-controlled peaks occur at nearly the same potential.[46]

Here, only the desorptive peak from the interface to the organic phase is observed. The readsorption from the organic phase upon scan reversal seems to be hindered. This could be due to the formation of peptide-encapsulating micelles in the organic phase (reverse micelles), as has been proposed for the transfer of cytochrome *c* facilitated by bis(2-ethylhexyl) sulfosuccinate.[47] Thus, once the desorption of the complex takes place, the association of the charged peptides in the organic environment with many phospholipid molecules occurs and the peptide–DPPC

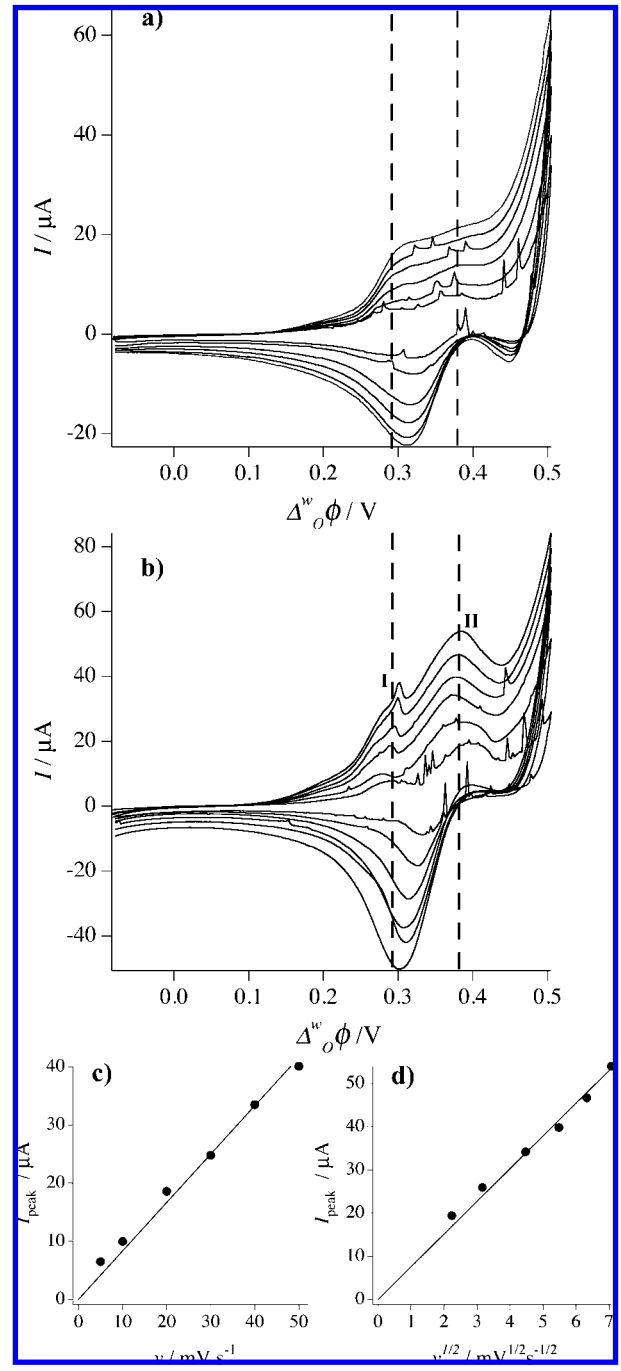


Figure 2. Ion-transfer voltammograms obtained at the interface formed between DCE in the presence of DPPC ($x = 10\ \mu M$) and an aqueous solution of AngIII (a) 100 and (b) 200 μM at scan rates of 5, 10, 20, 30, 40, and 50 $mV \cdot s^{-1}$. The dependencies of the peak currents for the signals labeled as I and II in (b) are presented in (c) and (d), respectively.

complex may change into a stable reverse micelle by association of complex molecules or fusion with other reverse micelles. Taking into account the hydrophobic shell presented by the stable reverse micelles, these are unlikely to be readsorbed again at the water–DCE interface and in consequence no significant readsorption electrochemical signals can be observed upon scan reversal.

Thus, the proposed mechanism for the peptide transfer across the interface consists of basically three steps, as follows:

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$$DPPC_{(o)} \rightleftharpoons DPPC_{(ads)}$$

$$n DPPC_{(ads)} + AngIII^{2+}_{(w)} \rightleftharpoons [DPPC_n - AngIII]^{2+}_{(ads)}$$

$$[DPPC_n\text{-}AngIII]^{2+}_{(ads)} \rightleftharpoons [DPPC_m - AngIII]^{2+}_{(o)} \quad (1)$$

Where the adsorption of the phospholipid dissolved in the organic phase, denoted as (o), at the interface, denoted as (ads), are comprised as well as the interfacial complexation between DPPC and $AngIII^{2+}$. The thermodynamic analysis of the lipid adsorption at the interface taking into account the interfacial complex formation and facilitated ion transfer has been performed by Samec et al.[48] using the Damaskin's adsorption model of a compound adsorbed in two different forms.[49] At the same time, Yoshida et al.[50] estimated the association constants between dioleoyl L-α-phosphatidylcholine and different metallic cations by assuming that the desorption potential, evaluated as the onset of the increase in the interfacial tension in electrocapillary curves, is dictated by the half-wave potential for the facilitated ion transfer by the phospholipid. Indeed, Marecek et al.[51] have also shown that the maximum surface coverage of the complex formed between protons and phosphatidylcholine occurs near to the desorptive peak potential. Thus after increasing the potential beyond the peak potential, the surface excess concentration of the complex will decrease. So that it can be obtained for the 1:1 complex:

$$\Delta\phi_{desorp} \approx \Delta\phi^{0\prime}_{AngIII^{2+}} - \frac{RT}{2F}\ln\left(K_c^o[AngIII^{2+}]\frac{D^{1/2}_{AngIII\text{-}DPPC^{2+}}}{D^{1/2}_{AngIII^{2+}}}\right) \approx \Delta\phi^{0\prime}_{AngIII^{2+}} - \frac{RT}{2F}\ln(K_c^o[AngIII^{2+}]) \quad (2)$$

where $\Delta\phi_{desorp}$ represents the desorption potential of the complex, $\Delta\phi^{0\prime}_{AngIII^{2+}}$ the standard ion-transfer potential of the free peptide that can be approximated to its half-wave transfer potential, easily determined from the cyclic voltammograms presented in Figure 1. K_c^o stands for the complexation constant in the organic phase, $[AngIII^{2+}]$ the bulk concentration of the peptide, and D the diffusion coefficient. In such a way, the complexation constant was determined to correspond to $5.2 \times 10^4\ M^{-1}$.

LeuEnk−DPPC System. For these experiments, AngIII was replaced by LeuEnk in the electrochemical cell in order to obtain electrochemical evidence of its interaction with a lipidic monolayer at the water−DCE interface.

First, cyclic voltammograms were obtained only in the presence of LeuEnk. As can be seen from Figure 3, no transfer of any charged species obtained in the presence of the peptide was evident (dotted line). As a matter of fact, according to its titration curve (see Figure S-2b, SI), this peptide has a zero net charge at a pH interval between 4 and 8, so that no appreciable ion-transfer electrochemical signals should appear. However, when the phospholipid is added to the electrochemical cell, the current increases in an appreciable quantity at potentials above 0.1 V, suggesting the existence of two broad signals. It is rather surprising to

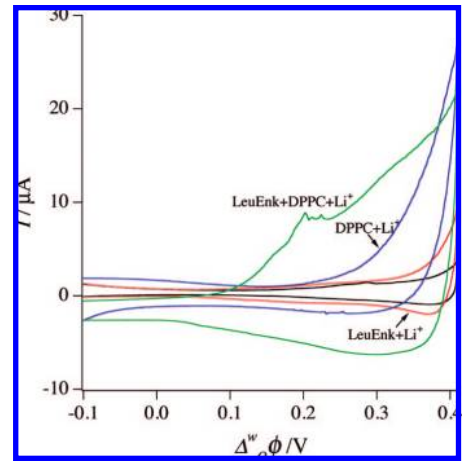
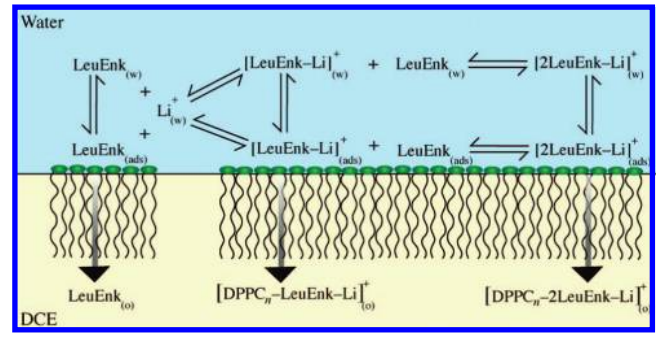


Figure 3. Comparison between the ion-transfer voltammogram obtained for an aqueous solution of LeuEnk ($y = 1000\ \mu M$) in the absence (blue line) and in the presence (green line) of DPPC ($x = 10\ \mu M$). The voltammograms obtained for the supporting electrolyte in the absence (black line) and in the presence of DPPC (red line) are also presented. Scan rate, 50 mV s^{-1}.

Scheme 3. Possible Mechanism for the LeuEnk Transfer Interactions with Lithium and a Monolayer of DPPC at the Water−DCE Interface



measure significant positive currents for this process, even when the pH of the aqueous phase is ~5, i.e., when both DPPC and LeuEnk have a net zero charge. These results can be explained by taking into account not only that at high concentrations of LeuEnk the lipid membranes may become unstable[11] but even more important that alkali metal ions can effectively interact with a wide variety of biologically important peptides, including LeuEnk, yielding charged complexes.[52,53] Thus, in the present case, the interactions between LeuEnk and DPPC lead to the adsorption at the phospholipid monolayer of charged complex species, either previously formed in solution between Li^+ and the free peptide or formed directly at the interface, which is followed by the desorption of these charged complexes. Hence, ion-transfer currents are observed at lower potentials in the presence of DPPC and LeuEnk. It has also to be taken into account that the partition of the neutral peptide can also occur as well as the formation of neutral complexes (Scheme 3). Nevertheless, no experimental evidence of these species is presented due to the fact that neutral compounds cannot be detected either by voltammetry at the ITIES or by BESI-MS.

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Again, the peptide concentration and the scan rate were evaluated (Figure 4). Two well-defined signals can be observed at low scan rates and they are shifted and broadened as the scan rate increases. This is the reason why voltammograms obtained at 0.05 $V \cdot s^{-1}$ and presented in Figure 3 in the presence of LeuEnk and DPPC (green line) exhibit two overlapped broad signals. According to Figure 4a, the currents obtained for both signals appear like interface-controlled processes rather than diffusion-controlled processes, being approximately proportional to the scan rate. On the other hand, after varying the peptide concentration (Figure 4b), it is evident that the ratio between the currents obtained for both signals changes as the concentration does (Figure 4c). Basically, the appearance of the signal at ~0.26 V is attributed to the dimerization of LeuEnk in solution.

The dimerization reaction of this peptide has already been reported[54,55] and could enhance not only the stability of the peptide in hydrophobic environments but also its interaction with the phospholipid monolayer. In the case under study, when the concentration of LeuEnk is increased the amount of the dimer increases too. Consequently, due to its extended antiparallel dimer structure,[56] its hydrophobicity, as well as those of its complexes with DPPC, is increased making it more suitable for being adsorbed at the monolayer. The final outcome of the dimerization process is therefore an enhanced interaction with the monolayer as well as the formation of more hydrophobic complexes whose transfer may occur at lower potentials than those observed for the complex with the monomer, present at ~0.38 V. In summary, the formation of different complexes, with different lipophilicities, leads to changes in the Gibbs energy of adsorption that exerts a great influence on the energy required for the transfer, commonly a decrease, which in turn decreases the potential required for the facilitated transfer mediated by DPPC.

Spraying of Phospholipids and Peptides with a BESI Source. To interpret further the electrochemical data presented above, it is interesting to obtain complementary information in order to formulate possible pathways that could account for the experimental behavior. Thus, DPPC was first tested with the commercial ESI source in pure DCE at 200 μM. DPPC and its dimer were observed at m/z = 734.5 Th and m/z = 1467.9 Th, respectively. The dimer represents the most intense peak with an abundance value close to 1×10^6 cts. Equivalent results were obtained with the microchip described above in pure DCE (dual-channel microsprayer where only the line dedicated to organic solvent was filled).

DPPC in DCE was then analyzed with the dual-channel microsprayer where the other line was filled with acidified water (AcOH at 1%). The dimer is still the most abundant peak but the absolute intensity is above 6×10^7 counts, and the phosphocholine fragment is produced at m/z = 184.2 Th.[15] In such a configuration, the protons stemming from the aqueous phase can react with the phospholipids at the interface within the Taylor cone or within the expelled droplets. As a result, the mass spectra quality is greatly enhanced. Moreover, this configuration leads to the study of phospholipids in a liquid−liquid system without additional solvents such as methanol that are miscible with both phases and that eventually could alter significantly the results obtained.

In order to probe the applicability of this experimental setup to the monitoring of complexes formation with DPPC, it was first verified that basic amino acids, like lysine (Lys) in its cationic form[50] and hydrophobic species like the dipeptide Phe−Phe (FF) are capable of forming complexes with DPPC at m/z = 880.2 Th for DPPC−Lys and m/z = 1046.0 Th for DPPC−FF (see Figure S-4, SI).

Here we used a biphasic electrospray source as an additional tool for the identification of the species that could form at the liquid−liquid interface. The main advantage of this microchip emitter is to be able to form a liquid−liquid interface within the Taylor cone, where an emulsification induced by the electric field is likely to take place. The present system differs from the coaxial liquid jet electrospray with two immiscible liquids investigated by Loscertales et al., who observed the production of capsules (water droplets coated by thin shells of oil).[57] However, the BESI mechanism is still under study, and it is likely that a mixture of aqueous and organic droplets is formed where the classical ion evaporation process takes place. Another point that has to be remarked is that in order to obtain significant responses from the mass spectra some experimental conditions might drastically differ from those employed in liquid−liquid electrochemistry. Among the most affected experimental parameters we can find pH, phospholipid or peptide concentrations, ionic strength of both phases, etc. In consequence, special care must be taken when correlating the data obtained from liquid−liquid voltammetry with the signals observed in the mass spectra.

The mass spectrum for AngIII in water contacted with DPPC in the organic phase is shown in Figure 5. From the spectrum, it is possible to observe the signal of $[DPPC + H]^+$ at a m/z value of 734.4 Th as well as the corresponding ones for the ions $[AngIII + H]^+$ and $[AngIII + 2H]^{2+}$ at m/z values of 931.5 and 466.3 Th, respectively. The complex formation between DPPC and AngIII, marked as $[DPPC-AngIII + H]^+$ and $[DPPC-AngIII + 2H]^{2+}$ in the spectrum, was also confirmed and their corresponding signals can be appreciated at m/z values of 1664.7 and 833.1 Th, respectively. This confirms the existence of complexes between AngIII and DPPC observed above by voltammetry.

On the other hand, the identification of DPPC dimers from the spectra was also made possible with the BESI sprayer, so that peaks for species like $[2DPPC + H]^+$ at m/z = 1467.9 Th and $[2DPPC-AngIII + 2H]^{2+}$ at m/z = 1199.6 Th are observed. The presence of these signals confirms the ability of the phospholipid to form dimers. This type of complexation reaction in which more than one complexing molecule are involved could constitute the initial steps of an early stage of the reverse micelles formation process; a process that has been identified as responsible for protein extraction in organic solvents and in our particular case could account for the hindering of the readsorption of the transferred peptides, as mentioned and discussed above in the text.

Furthermore, the relatively weak abundance values of the noncovalent complexes obtained for DPPC and AngIII are prob-

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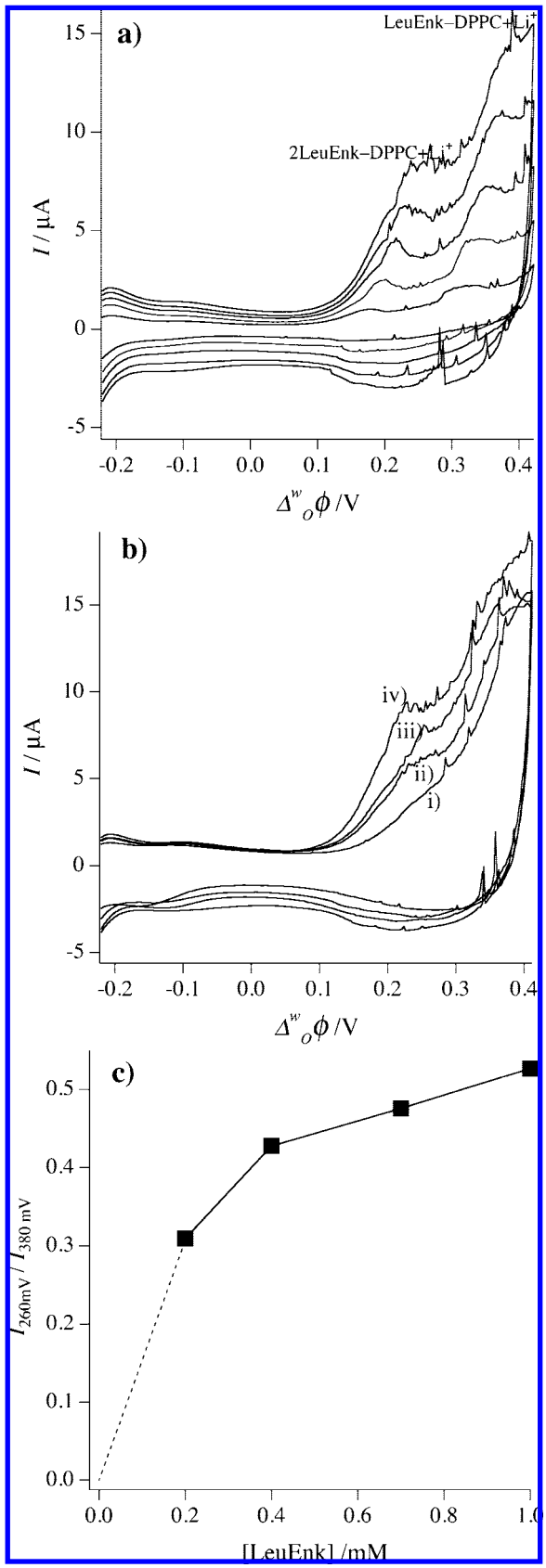

Figure 4. Ion-transfer voltammograms obtained at the interface formed between DCE in the presence of DPPC (x = 10 μM) and an aqueous solution of LeuEnk (a) 700 μM at scan rates of 5, 10, 15, 20, and 25 mV·s^{-1}. In (b), the cyclic voltammograms at 25 mV·s^{-1} for y = 200 (i), 400 (ii), 700 (iii), and 1000 (iv) μM are presented. (c) Ratio between the magnitude of the currents obtained for the electrochemical responses presented in (b) at 260 and 380 mV, respectively.

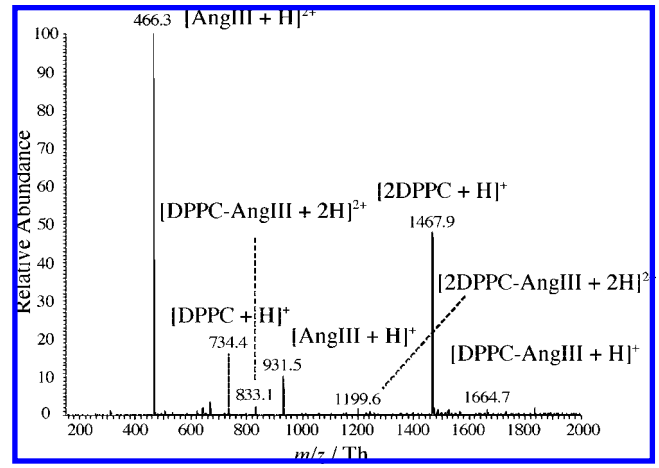

Figure 5. Mass spectrum of the reaction between DPPC 200 μM in DCE and AngIII 200 μM in acidified water (1% acetic acid) obtained with a BESI source coupled to the ion trap mass spectrometer.

ably not only related to the poor ionization efficiency or low concentration of these complexes but also to the high ionization efficiency of AngIII itself, which consequently reduces the signal of the other ions. Nevertheless, the MS/MS of DPPC−AngIII was easily obtained at 25% of collision energy with a high abundance. The fragmentation clearly produced only AngIII and DPPC ions.

Analogously, an aqueous solution of LeuEnk 200 μM containing AcOH 1% was put in contact with an organic solution and sprayed with the same experimental setup as described above (see Figure S-5, SI). In general, the complex formation was evidenced as well as the formation of the LeuEnk dimer. However, due to the fact that no lithium cations were present in the aqueous solution, the cationization of the peptide was not observed. To probe the existence of the complex not only between LeuEnk and lithium but also between the latter and DPPC, similar experiments were conducted in an ESI-TOF mass spectrometer infusing in the channel dedicated for the aqueous phase a solution of 1 mM LiCl instead of an acidic solution (AcOH 1%). Thus, compounds with m/z values beyond 2000 Th were accessible, and at the same time, the reactivity of the peptide toward lithium and DPPC was also tested.

The results obtained from these experiments are summarized in Figure 6. As can be seen from this figure, the species observed before in the absence of LiCl such as $[DPPC + H]^+$, $[2DPPC + H]^+$, $[LeuEnk + H]^+$, $[2LeuEnk + H]^+$, $[DPPC-LeuEnk + H]^+$, and $[DPPC-2LeuEnk + H]^+$ appear again. In addition to these, intense peaks that represent the signals obtained for the species in which the proton is replaced by a lithium cation are presented for all those just mentioned. The m/z values of these compounds are indicated in Figure 6. In fact, when the mass interval is restricted to m/z values between 1260 and 1340 Th, the appearance of the complex between DPPC and LeuEnk carrying a positive charge from either protons or lithium, namely, $[DPPC-LeuEnk + H]^+$ at 1290.2 Th or $[DPPC-LeuEnk + Li]^+$ at 1296.2 Th, is evident.

According to this, the hypothesis formulated around the cationization of the peptide with lithium in solution or at the interface is here corroborated and explains the positive currents registered in the cyclic voltammograms for LeuEnk in the

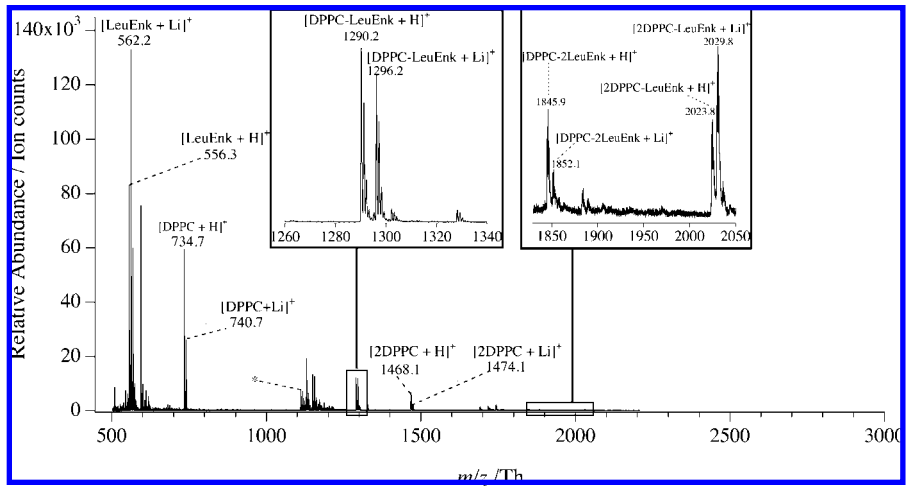

Figure 6. Mass spectrum of the reaction between DPPC 200 μM in DCE and LeuEnk 200 μM in acidified water containing LiCl 1 mM obtained with a BESI source coupled to the time-of-flight mass spectrometer. (*) represents $[2\text{LeuEnk} + \text{Li}]^+$.

presence of DPPC (Figure 3). It can also be appreciated from the same figure that, after zooming in the region between m/z values of 1800 and 2050 Th, the peaks for the lithium-containing complexes $[\text{DPPC}-2\text{LeuEnk} + \text{Li}]^+$ and $[2\text{DPPC}-\text{LeuEnk} + \text{Li}]^+$ are detected at 1852.1 and 2009.8 Th, respectively. The existence of these species corroborates the proposed mechanism as it proves not only that the LeuEnk dimer is also reactive toward the complex formation with lithium and DPPC but also that, as observed for AngIII, the lipidic dimer is capable of forming complexes with the cationized LeuEnk peptide. As mentioned above, this experimental fact allows us to propose the reverse micelles formation at the organic phase.

An interesting phenomenon here observed is the higher intensity obtained for the LeuEnk complexes, both in the absence and in the presence of LiCl, when compared with the relative abundance of the DPPC−AngIII complex, which can be explained by either the spray quality or the high production of the former ion. As a matter of fact, the hydrophobic part of these two peptides (presented in a higher extent for LeuEnk) seems to induce stabilizing effects over the complexes. Of course this effect is much more accentuated for the amino acid and the dipeptide tested.

Finally, the lability of the complexes as a function of the heated capillary temperature was studied and is presented in Figure 7. The trends for DPPC−Lys and DPPC−FF (Figure 7a) are to decrease rapidly and completely disappear at temperatures beyond 150 °C, whereas the relative abundances of the peptides show parabolic shapes and are still observed at 200 °C. This trend can be considered from two different perspectives. First, it is associated with the stability of the complexes with the temperature. Second, it was also observed that the ionization efficiency of compounds with low m/z values decreases when the temperature of the heated capillary was increased. However, since the m/z of the complexes analyzed here are between 880 and 1289 Th, the second effect can be neglected. So that noncovalent bonds (dipole−dipole interactions, van der Waals forces, etc.) are weak and their abundance apparently enhances the stability. Nevertheless, an increment in the temperature can cleave this type of bonds. Therefore, very weak interactions as those in the complexes DPPC−Lys and DPPC−FF might be easily destroyed by raising the heated capillary temperature, explaining the absence of any signal of these complexes at, for instance, 200 °C.

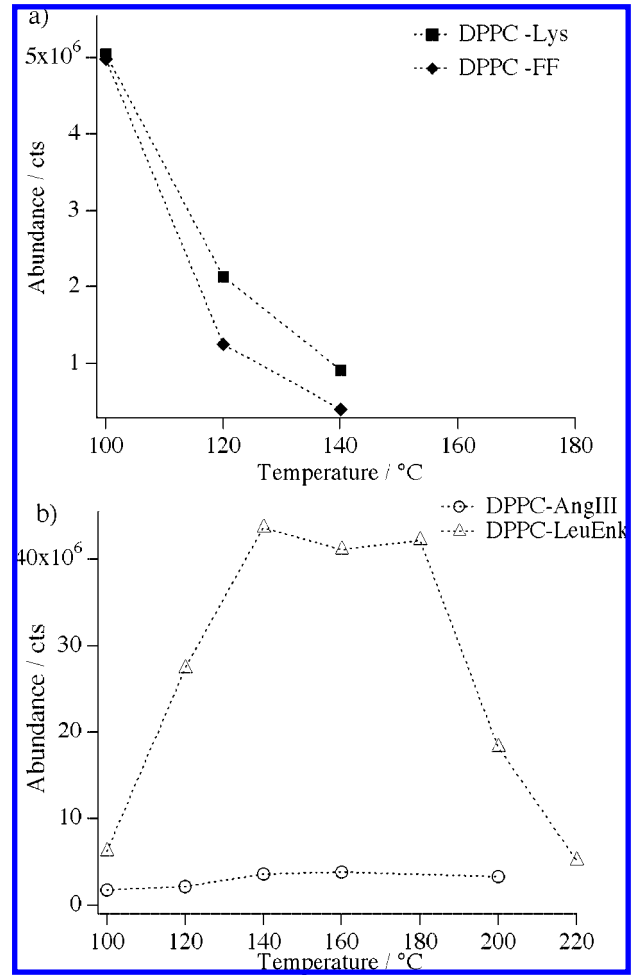

Figure 7. Relative abundance of the complexes as a function of the heated capillary temperature. (a) DPPC−Lys and DPPC−FF and (b) DPPC−AngIII and DPPC−LeuEnk. Beyond 150 °C, DPPC−Lys and DPPC−FF are not observed.

In particular, for the case of the two peptides, their complexes with DPPC showed some stability over the whole temperature range of the heated capillary tested. Indeed, the exhibited

parabolic shape is typical and can be explained by the balance between the quantity of gas-phase ions produced and the transmission to the analyzer. The evaporation process that produces gas-phase ions is not favored at low temperature, but the ions' mobility is thus low enough to allow them to fly in the MS. On the contrary, at high temperature, the ions can be lost on the wall of the apparatus but the desolvation process is consequently enhanced.[58] As can be expected from the first experiments conducted with lysine and FF, by increasing the length of the peptide, the complexes formed with DPPC should become more stable due to the extended hydrophobic interactions. Indeed, the extent of these interactions should be greater for LeuEnk than for AngIII. These hydrophobic residues can increase the complex stability. Leu-enkephalin is known to interact with lipid membranes[59] and Carpenter et al. have shown that angiotensin II (that differs from AngIII only by the presence of an aspartic acid at the N-terminus) adopts a hairpin shape in a phospholipid micelle environment,[60] which corroborates once again the results presented herein.

CONCLUSIONS

In the present work, we have shown electrochemical evidence for the formation of peptide−lipid complexes adsorbed at the interface between DPPC and AngIII and LeuEnk. The complexation is likely to involve both Coulombic and van der Waals interactions. In the case of AngIII, the peptide−lipid complex can transfer to the organic phase resulting in an adsorptive prepeak in the voltammetry. However, for LeuEnk, the interaction mechanism seems to be ruled in the first place by Coulombic interactions, either with the lipid adsorbed at the interface or with the cations present in solution, and in the second place by hydrophobic interactions with the lipid.

Conformational structures are also proposed to play a vital role in the interaction since LeuEnk dimer also interacts with an adsorbed DPPC monolayer making possible the differentiation between the current signals coming from the desorption of the lipidic monolayer that interacts with the monomer and the dimer of LeuEnk. Finally, the successful coupling of liquid−liquid electrochemistry and BESI-MS leads to the formulation of mechanisms in which Coulombic and hydrophobic interactions can be accounted for.

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SUPPORTING INFORMATION AVAILABLE

The calculated charge values for AngIII and LeuEnk as a function of pH. Details concerning the BESI source as well as the mass spectrum of DPPC analyzed with the commercial ESI source and the BESI and the mass spectra of DPPC-Lys and DPPC-FF. This material is available free of charge via the Internet at http://pubs.acs.org.

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