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# A study of melittin, motilin and galanin in reversed micellar environments, using circular dichroism spectroscopy

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**Abstract**

Circular dichroism spectroscopy has been used to study the behaviour of the cytolytic peptide melittin, the intestinal peptide hormone motilin (porcine) and the neuropeptide galanin (porcine) in various reversed micellar systems. The micellar systems used contained sodium dodecyl sulphate, bis(2-ethylhexyl) sulfosuccinate, *n*-dodecyltrimethylammonium chloride or polyoxyethylene(7) lauryl ether. Various structural changes of the peptides, induced either by varying the water content or the surface charge of the reversed micelles, could be monitored. Melittin has in all micellar systems a large amount of $\alpha$-helix, and is almost unaffected by both water content and the surface charge of the reversed micelles. Motilin on the other hand attains an $\alpha$-helical structure at low water content only. The surface charges seem to be of importance for the association between motilin and the hydrated reversed micellar surface. Galanin has the most complicated behaviour with a large dependence on surface charge and with a water content dependence which varies with the surfactant used. Stabilization of $\alpha$-helical secondary structures was only seen in negatively charged reversed micelles. These observations indicate a specific interaction between galanin and surfactant, probably of electrostatic nature.

*Keywords:* Peptides; Melittin; Motilin; Galanin; Reversed micelles; Circular dichroism spectroscopy

## 1. Introduction

There are a large number of short, biologically active peptides, whose secondary structures are strongly dependent on the environment [1]. These peptides often carry out their function by interaction with membrane-bound receptors and/or phospholipid membranes. In a more hydrophobic environment, better defined secondary structures are generally observed, usually quite different from the more random coil structures seen in aqueous solution [2]. These better defined structures may mimic the functional ones, existing when the peptide interacts with a receptor or a biomembrane. Three peptides which share some of these characteristics but with rather different functions are melittin, motilin and galanin. The aim of the present study was to investigate how the hydration of the peptides as well as a hydropho-

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bic environment affects the secondary structure stabilization of these peptides.

Melittin, a major constituent of bee venom, is a 26 amino acid long peptide with the sequence GIGAVLKVLTTGLPALISWIKRKRQQ-$NH_2$. It has 20 predominantly hydrophobic amino acids in its N-terminal part and 6 either polar or charged residues in the C-terminal part [3]. At neutral pH it has a net charge of +6. It has the ability to disrupt both natural and synthetic phospholipid membranes [4] and is known to be a strong hemolytic agent [3]. It is also believed to work as an activator to phospholipase $A_2$ [5] and to have fusogenic activity [6]. In aqueous solution melittin exists as a monomer or as a tetramer [7], but when it binds to a biomembrane, it folds into a monomeric $\alpha$-helical rod of amphiphilic character with a bend at position 11 and 12 [8,9]. This wedge-like structure is located at the outer surface of the membrane [10]. Under the influence of a membrane potential the peptide may, however, attain an $\alpha$-helical transmembrane conformation [4].

Motilin is a gastrointestinal peptide hormone which consists of 22 amino acids. The sequence of porcine motilin, FVPIFTYGELQRMQEKERNKGQ [11], shows a mainly hydrophilic character, although the five first amino acids are non-polar. At neutral pH this peptide has a net charge of +1. The N-terminal part of the peptide binds to a membrane-bound receptor in the smooth muscle tissue of the gastrointestinal tract [12,13], where the hormone stimulates gastrointestinal motor activity [14]. Its structure in an aqueous solvent with 30% 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) is known from nuclear magnetic resonance (NMR) studies [15], and shows that motilin displays an $\alpha$-helix from residue 9–19 and a wide turn from residue 3–6. These two structural elements are separated by a less ordered hinge-like structure around the tyrosine in position 7. In water the peptide attains a more random coil structure. Previous circular dichroism (CD) studies [16] have shown that motilin has almost the same secondary structure content in solvents with negatively charged vesicles and micelles as in 30% HFP.

Galanin [17] is a 29 amino acid long peptide (30 in humans [18]), which in the porcine species has the sequence GWTLNSAGYLLGPHAIDNHRSFHDKYGLA-$NH_2$. Galanin has several important functions in the neuroendocrinic system [19,20], where its biological functions are mediated via a membrane-bound specific high-affinity receptor [21]. Previous structural studies have demonstrated that galanin, which has a net charge of +2 (neutral in humans) at pH 7, has a low degree of $\alpha$-helical secondary structure in water [22–24] and attains more $\alpha$-helical structure in systems of negatively charged vesicles and micelles and also in fluorinated alcohols [23,24]. The three-dimensional solution structure has been determined by NMR in 100% 2,2,2-trifluoroethanol (TFE) [25,26], and shows that the peptide has two $\alpha$-helices separated by a bend at the only proline residue at position 13.

Previous structural studies on these peptides have often utilized lipid vesicles and detergent micelles in aqueous solution to mimic their functional environment. In the present study, the secondary structures of the peptides were determined in various reversed micellar systems, using CD spectroscopy. An interesting aspect of using reversed micelles as a model for the functional environment of the peptides is that it gives the possibility to vary the water content within the micellar aggregates (for reviews, see [27,28]). An important parameter in this context is the molar ratio of water to surfactant, expressed as $w_0 = [H_2O]/[\text{surfactant}]$. The water pool in a reversed micelle solvent consists of at least two distinct populations of water molecules with different properties: (i) the interfacial, surfactant-bound water where surfactant counterions (if present) are most likely localized [29] and (ii) the bulk water which appears when $w_0$ is increased and the hydration of the surfactant has become complete.

In the micellar systems studied here, structural changes of the peptides were induced by varying the amount of water in the systems, i.e. at different $w_0$. We have also investigated how the head group charges and the presence of counterions affect the stabilization of secondary structure in the peptides.

## 2. Material and methods

### 2.1. Materials

1-Hexanol (synthesis grade), *n*-heptane (Uvasol grade), isooctane (Uvasol grade) and sodium chlo-

ride (NaCl) were purchased from Merck, Darmstadt. Bis(2-ethylhexyl) sulfosuccinate (AOT) was purchased from Fluka, polyoxyethylene(7) lauryl ether ($C_{12}E_7$), 2,2,2-trifluoroethanol (TFE) and melittin (quality: approx. 85%) from Sigma, sodium dodecyl sulphate (SDS) from Kebo, *n*-dodecyltrimethylammonium chloride (DoTAC) from TCI, Tokyo Kasei and finally 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) from Avanti Polar Lipids, USA.

Porcine galanin was synthesized, purified and characterized as previously described [30]. Porcine motilin was either obtained from natural sources (sample kindly provided by professor Viktor Mutt, Karolinska Institutet, Stockholm) or synthesized on solid phase using *t*-Boc chemistry [31]. Both motilin samples gave indistinguishable results. Aqueous DOPG vesicles were prepared as previously described [16].

### 2.2. Preparation of samples of reversed micelles

A 0.2 M SDS reversed micellar solution was prepared by adding a mixture of *n*-hexanol and *n*-heptane (17:3, v/v) to dry pre-weighed quantities of SDS. In order to obtain a clear solution pure water was added to a $w_0$ value corresponding to 6.1 and the solution was heated to 80°C for 15 min and shaken.

The preparation of the 0.06 M AOT reversed micellar solution, $w_0 = 0$, was done by dissolving an appropriate amount of AOT in *n*-heptane.

The 0.2 M DoTAC reversed micellar solution, $w_0 = 0$, was prepared by adding a mixture of *n*-hexanol and *n*-heptane (3:22, v/v) to dry pre-weighed quantities of DoTAC, followed by heating at 50°C.

A 0.115 M $C_{12}E_7$ reversed micellar solution, $w_0 = 0$, was prepared by adding an appropriate volume of $C_{12}E_7$ to a mixture of isooctane and *n*-hexanol (25:4, v/v).

The peptides were introduced into the reversed micelle solutions by adding a small volume (2–4 $\mu$l) from a concentrated peptide stock solution to the various reversed micelle solutions (usually 500 $\mu$l), yielding final peptide concentrations between 20 and 80 $\mu$M. The obtained solutions were titrated with pure water and CD spectra were recorded. Peptide stock solutions were prepared by adding appropriate amounts of water (melittin and motilin) or TFE (galanin) to a lyophilized dry powder of the peptides. The stock solution concentrations were 17 mM for melittin, 20 or 33 mM for motilin and 10 mM for galanin.

### 2.3. Circular dichroism spectroscopy

CD spectra were recorded using a Jasco-720 spectropolarimeter and 0.5-, 1- or 2-mm cuvettes. Measurements in the neutral $C_{12}E_7$ were performed at 4°C, all the others at 25°C. The parameters used were a bandwidth of 1 nm, a 2 s response time, a resolution of 0.2 or 0.5 nm and a scan speed of 10 nm/min. The acquired spectra were the average of two or three continuous scans, usually measured between 190 and 250 nm, and corrected with respect to baseline and for dilution. In a few cases only one scan was used. The spectra have been moderately noise reduced and are presented in units of mean residue molar ellipticity. The relative helicity of the peptides was calculated from the mean residue molar ellipticity at 222 nm as previously described [16]. There are, however, some uncertainties in the concentration determination of the peptides, partly due to the uncertainties in adding a small volume of highly concentrated peptide stock solution to a large volume of surfactant solution, and also due to light scattering and high absorbance from the surfactants which makes absorbance measurements less accurate. As a consequence, quantitative comparisons of the data, other than within each titration series, should be done with caution.

## 3. Results and discussion

The studied peptides all have their physiological activities in the vicinity of lipid membranes, either by membrane binding and/or by interacting with membrane-bound receptors. Their rather unstructured conformations in aqueous solution transform into well-defined $\alpha$-helical structures in the lipidic environment as has been shown previously [8,9,16,23,24]. We have now studied the secondary structure stabilization of the peptides in four differently charged reversed micellar systems at different levels of micellar hydration ($w_0$). The AOT and SDS surfactants

are both anionic, while $C_{12}E_7$ is neutral and DoTAC is cationic. The most widely studied and characterized surfactant is AOT [27–29,32,33], but studies on surfactants similar to the others have also been performed [33,34]. Various molar ratios of water to surfactant, $w_0$, were used to investigate the effect of hydration of the peptide on its secondary structure and the nature of the peptide–surfactant interactions. The three peptides studied show significantly different behaviour in their interactions with the reversed micelles, and their individual behaviours may have functional importance for their physiological activities.

In the neutral $C_{12}E_7$ micellar system at $w_0$ less than 5, the solubility of the peptides was poor. At higher $w_0$ values the solubility increased making it possible to perform CD measurements.

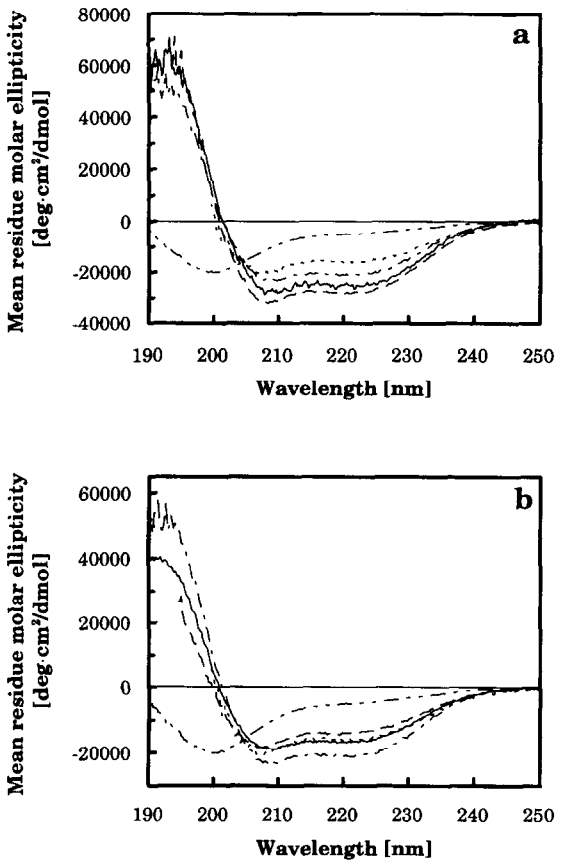


Fig. 1. CD spectra of melittin dissolved in (a) water (·····) and reversed micelles of the surfactants AOT at $w_0 = 20$ (—), $C_{12}E_7$ at $w_0 = 35$ (— — —), DoTAC at $w_0 = 20$ (– – –) and SDS at $w_0 = 20$ (-·-·-); (b) water (·····), normal aqueous micelles of DoTAC (— — —) and SDS (—) at a concentration of 0.1 M, and in reversed micelles of DoTAC (– – –) and SDS (-·-·-) at $w_0 = 20$.

Table 1
Estimated amount of α-helix in percent of the peptides in water and four reversed micellar solvents

| | $H_2O$ | AOT [a] | SDS [b] | $C_{12}E_7$ [c] | DoTAC [d] |
|---|---|---|---|---|---|
| Melittin | 21 | 70 | 59 | 75 | 47 |
| Motilin | 16 | 19 | 20 | 21 | 16 |
| Galanin | 11 | 16 | 16 | – [e] | – [f] |

[a] Measurements on melittin, motilin and galanin in AOT performed at $w_0$ values of 20, 20.3 and 30, respectively.
[b] Measurements on melittin, motilin and galanin in SDS performed at $w_0$ values of 20, 24 and 20, respectively.
[c] Measurements performed at $w_0 = 35$.
[d] Measurements performed at $w_0 = 20$.
[e] Not possible to evaluate due to aggregation problem.
[f] Not possible to dissolve.

### 3.1. Melittin

Fig. 1a shows that the stabilized secondary structures of melittin are qualitatively very similar in all the reversed micellar systems. It attains a high degree of α-helicity in these systems compared to the structure in water, Table 1. Varying the $w_0$ values in each system did not change the observed CD spectra. This absence of sensitivity to water content is further illustrated in Fig. 1b, where the strong similarities between the CD spectra seen in reversed micelles and those seen in normal aqueous micellar solution of the same surfactants are shown.

Taken together, these observations suggest a localization of the peptide to the hydrocarbon phase or the interfacial water region, where binding or close contact between peptide and surfactants occurs. The strong peptide–surfactant interactions are marginally affected by the micellar surface charge and almost independent of the water content of the micelles. This behaviour suggests that the hydrophobic interactions are highly important and reflects the potent membrane binding ability of melittin, which is the basis for its hemolytic capacity.

### 3.2. Motilin

Motilin behaves quite differently from melittin. The most striking observation is a strong dependence

on water content in the various reversed micelles. As an example, the CD spectra of motilin in AOT reversed micelles, where $w_0$ is varied between 3.7 and 20.3, is shown in Fig. 2. At low $w_0$ motilin has a pronounced $\alpha$-helical structure but as the water content increases the peptide attains a less ordered structure, similar to the one observed in aqueous solvent. At $w_0$ larger than 10–15, no further changes of the structure are seen. It has previously been shown that the hydration of the head group surface in AOT reversed micelles becomes complete at $w_0 \geq 10$ and that a bulk water pool is created [32]. The behaviours of the peptide in SDS and DoTAC are similar to what is observed in AOT micelles. In neutral $C_{12}E_7$ micelles motilin aggregated at low $w_0$. The peptide was solubilized at intermediate $w_0$ values ($w_0$ between 5 and 10) where a rather well structured conformation was seen. As $w_0$ is increased further motilin gradually attains a more random conformation towards the structure seen in water.

Our data suggest that motilin, in micellar systems with $w_0$ higher than 10–15, is preferentially located in the bulk water pool at the center of the micelles, independent of headgroup charges. As a consequence, the peptide attains a rather unstructured conformation, similar to that in water (see Table 1). Since previous studies [16] have shown that motilin has an $\alpha$-helical structure in the presence of negatively charged micelles and vesicles in aqueous solution, these results seem to be contradictory. However, the localization of motilin to the bulk water pool in present experiments can be explained by a relatively low counterion dissociation leading to a high local concentration of counterions close to the surface of the micelle. The net charge of the micellar surface is thereby decreased, accompanied by a reduction of the electrostatic interactions between the surface and the peptide. Measurements on AOT reversed micelles have shown that the concentrations of counterions could reach 5 M in the interfacial water region [29]. This explanation is also supported by a titration series, as can be seen in Fig. 3, where salt has been added (up to 1 M) to normal negatively charged aqueous DOPG vesicles with a reduction of $\alpha$-helicity of the peptide as a result.

Previous studies have shown that the surface charge of aqueous micelles and vesicles is of impor-

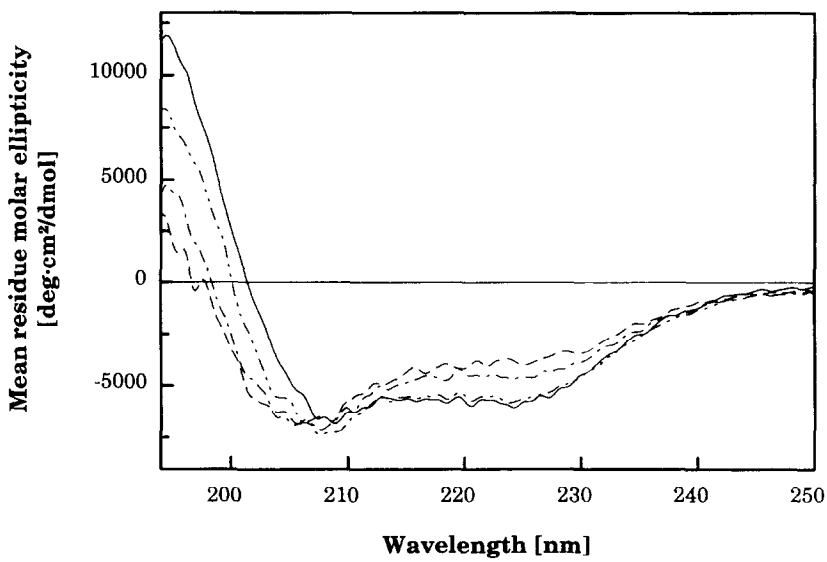


Fig. 2. CD spectra of porcine motilin in reversed micelles of AOT at various $w_0$. $w_0 = 3.7$ (—), $w_0 = 7.4$ (-·····-), $w_0 = 11.1$ (-·-·-) and $w_0 = 20.3$ (— — —).

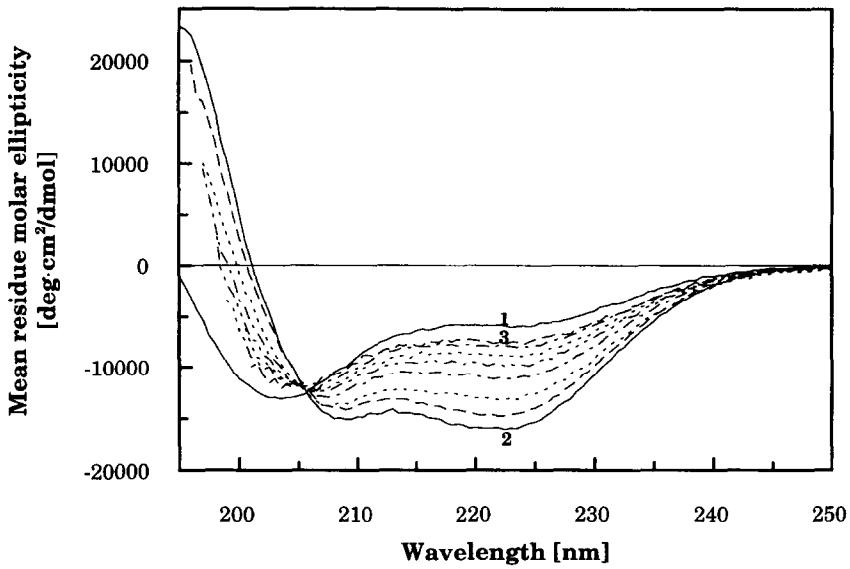


Fig. 3. CD spectra of porcine motilin in (1) pure water and in aqueous 0.1 mg/ml DOPG vesicle solutions at NaCl concentrations of (2) 0, 50, 100, 150, 200, 300, 500 and (3) 1000 mM.

tance for structure stabilization of motilin [16]. Based on these and present observations, we propose the following mechanism for structural stabilization. Motilin associates with lipid surfaces due to electrostatic forces. At this peptide–lipid interface, the exclusion of water facilitates intramolecular hydrogen

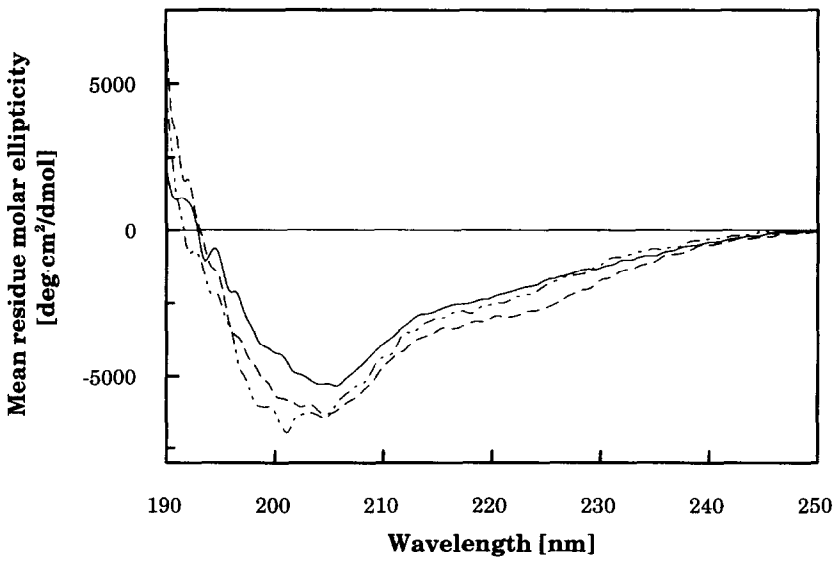


Fig. 4. CD spectra of porcine galanin dissolved in normal aqueous micelles of SDS (—) and in reversed micelles of SDS at $w_0 = 7$ (· · · · · ·) and $w_0 = 20$ (— — —).

bonds and hence secondary structure stabilization of the peptide.

### 3.3. Galanin

The third peptide, porcine galanin, has again a completely different behaviour. It was not possible to dissolve galanin in positively charged DoTAC micelles at any $w_0$ (0–30). In neutral $C_{12}E_7$ micelles, the galanin solution aggregated at low $w_0$. As more water was added, the peptide gradually dissolved ending in a conformation with a CD spectrum similar to what is observed in an aqueous solvent. We therefore conclude that the peptide preferentially populates the bulk water pool in $C_{12}E_7$ micelles. The negatively charged SDS and AOT reversed micellar solvent systems both stabilized more $\alpha$-helical secondary structures, as compared to the structure in water, cf. [23,24] and Table 1. As illustrated in Fig. 4, these structures are almost insensitive to different $w_0$. This indicates a localization of the peptide to the lipid phase or interfacial water close to the membrane in these systems.

Previous studies, in aqueous solvents, have shown that galanin is unaffected by zwitterionic vesicles as compared to water, but that it attains a well-defined, somewhat $\alpha$-helical, secondary structure in the presence of negatively charged vesicles and micelles [23,24]. The behaviour of galanin in neutral and negatively charged reversed micellar systems agree well with these observations. Judged from the spectra in Fig. 4, the induced secondary structures of galanin in reversed and normal aqueous micelles of SDS are actually very similar.

The obtained data suggest that the electrostatic interactions are very important for the solubilization of galanin and should also contribute to its structure stabilization. The high concentration of counterions within the negatively charged micelles does not seem to affect the preferred localization and induced secondary structure of the peptide. These observations and the fact that galanin has a positive net charge indicate that the electrostatic interactions with the micellar systems seem to be both strong and specific. However, like in the case of motilin, exclusion of water at the peptide–lipid interface may also occur and favour intramolecular hydrogen bonds in the peptide, resulting in a further stabilization of an $\alpha$-helical structure [35].

## 4. Conclusions

Motilin and galanin mediate their functions via membrane-bound receptors, whereas melittin acts directly on the membrane. Differences in their membrane interaction mechanisms are therefore expected and are in fact seen, not only between melittin versus motilin and galanin, but also to some extent when comparing motilin and galanin. From the results presented in this study together with previous observations the following three mechanisms emerge for stabilizing secondary structures of the peptides by biomembrane association:

(i) Melittin, with a net charge of +6, shows a strong membrane association, with a concomitant structure stabilization, almost independent of the charge of the lipid head group and the water content of the micelle. The association should have a significant contribution from hydrophobic interactions.

(ii) Motilin, which has a +1 net charge, relies mainly on electrostatic interactions to associate with the lipidic surface. The structure stabilization is accomplished by the exclusion of water at the peptide–lipid interface.

(iii) The structure stabilization of galanin, with a net charge of +2, seems to involve strong and specific interactions with the amphiphilic molecules, with a significant contribution from electrostatic forces.

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