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The interaction of the cell-penetrating peptide penetratin with heparin, heparansulfates and phospholipid vesicles investigated by ESR spectroscopy

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Abstract: An ESR investigation of the interaction of spin-labelled penetratin with heparin, heparansulfates and several phospholipid vesicle formulations is reported. Penetratin is a 16-aa peptide corresponding to the third helix of the Antennapedia homeodomain and belonging to the cell-penetrating peptide family. The present study shows that ESR spectroscopy can provide specific and reliable information about the mechanism of interaction of penetratin with polysaccharides and lipids, at a molecular level. The study showed that: (i) heparin and heparansulfates specifically interact with spin-labelled penetratin and promote peptide aggregation and concentration on their molecular surface; (ii) penetratin does not interact with neutral lipids, whereas it enters negatively charged lipid bilayers; (iii) cholesterol plays a negative effect on the insertion of penetratin into the lipid membrane; (iv) the interaction of penetratin with lipid vesicles is strongly dependent on lipid concentration. In a low lipid regime, penetratin associates with the polar heads of phospholipids and aggregates on the membrane surface; once the lipid concentration attains a threshold, the peptide enters the lipid bilayer. This step is characterized by reduced peptide mobility and partial disaggregation.

It has been shown that ESR spectroscopy is a valuable investigation tool in studies related to the still unclear mechanism of the internalization process.

Keywords: cell-penetrating peptide; trojan peptide; ESR spectroscopy; phospholipid vesicle; cell internalization

INTRODUCTION

Cell-penetrating peptide (CPP) vectors are a class of peptides able to translocate freely across plasma membranes with high efficiency, without compromising normal cellular functions.

They include a growing number of amino acid sequences; amongst them, TAT peptide, that is part of the HIV TAT transactivation protein, and penetratin (pAnt), a 16-amino acid fragment of the Antennapedia homeodomain, are the most widely studied.

The ability of these molecules to translocate bulky molecular cargoes across cell membranes without disrupting the lipid bilayer makes them appealing for drug delivery purposes.

In spite of the fact that the interaction of CPP with cell membranes (or model membranes) has been studied by a number of techniques, i.e. fluorescence, CD, NMR, ESR, PM-IRRAS, optical spectroscopy, flow cytometry and immunocytochemistry assays [1–16], the exact mechanism of cell entry has not yet been identified. The involvement of an endocytotic mechanism of internalization has long been a matter of discussion [17–20]. Recent studies have definitely proven that both endocytotic and non-endocytotic pathways are involved in the internalization of CPPs [2,19–22], moreover, it has been suggested that diverse types of peptides might follow different internalization pathways [2,19]. Several physico-chemical factors have been shown to play a role in the translocation process; e.g. amphiphilicity and the presence of both positively charged and hydrophobic residues (as Arg and Trp, respectively) in the sequence. Three mechanistic models have been proposed up to now [23 and references therein]. The first is based on the formation of inverted micelles inside the lipid bilayer [8,24–29]. The second implies an 'electroporation-like' permeabilization of the membrane, as the result of perturbations brought about by pAntp on the transmembrane electrical field in lipid vesicles [30]. The third is the so-called 'carpet-model' [23]: it was first suggested for translocation of antimicrobial peptides and then it was extended to CPPs. ESR spectroscopy was used as a tool to obtain detailed information about

Abbreviations: aa, amino acid; CD, circular dichroism spectroscopy; CPP, cell-penetrating peptides; ESR, electron spin resonance spectroscopy; FIA/MS, flow injection analysis/mass spectrometry; GAG, glycosaminoglycan; IR, infra-red spectroscopy; LC/MS, liquid chromatography/mass spectrometry; MTSL, 1-oxyl–2,2,5,5,-tetramethyl-Δ^3-pyrroline-3-methyl)-methanethiosulfonate spin label; NMR, nuclear magnetic resonance spectroscopy; PM-IRRAS, polarization modulation infrared reflection absorption spectroscopy; POPC, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-glycerol); PtdSer, 1,2-diacyl-sn-glycero-3-phospho-L-serine; τ_C, rotational correlation time.

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the interaction of penetratin with some components of cell membranes, at a molecular level; the interest of this study comes from its relation to the problem of peptide internalization.

The 16-aa penetratin peptide was used as a paramagnetic probe after adding a supplemental Cys residue at its *N*-terminal and conjugating it to a thiol-specific spin-label. The interaction of labelled penetratin with heparin, heparansulfates and phospholipid vesicles was investigated. The choice was due to the fact that heparin and heparansulfate proteoglycans are found on the surface of most cells [16,31]. Several papers suggest their involvement in the interaction of CPPs with cells [15,32]. As for phospholipid vesicles, they are a suitable model for cell membranes; several different vesicle formulations have been used in the present study.

Only two ESR studies have been published on the subject to date. In both cases, the technique was employed to highlight the reduced mobility of penetratin interacting with the lipid phase [7,9]. The present study is based on an original approach of the internalization topic by ESR spectroscopy and demonstrates that ESR provides an insight into the mechanism of interaction of CPPs with cell components, at a molecular level: this could be of great help in solving the puzzling challenge of the CPP's mechanism of action.

MATERIALS AND METHODS

Heparin sodium salt, heparansulfate sodium salt, POPG ammonium salt, POPC, PtdSer, cholesterol, sodium acetate, dithiothreitol and a pre-liposome formulation containing POPC, POPG and cholesterol were purchased from Sigma. MTSL was purchased from Toronto Research Chemicals.

Peptide Synthesis and Purification

The peptide was assembled on an Applied Biosystems Model 433A Peptide Synthesis System by conventional solid phase chemistry using a Fmoc/tBu strategy and purified on a reversed-phase C18 column with 10 μm beads after trifluoroacetic acid (TFA) cleavage/deprotection. The peptide was lyophilized twice and assessed for identity and purity by FIA/MS and LC/MS. The peptide sequence was CRQIKIWFQNRRMKWKK with the *C*-terminus blocked in the amide form.

Peptide Labelling

MTSL was dissolved in N,N-dimethylformamide (5 molar equivalents). The thiol-containing peptide (1 molar equivalent) was then added, followed by a 4 h incubation. Further processing and a purity check of the conjugate were performed as described above.

ESR Measurements

All ESR measurements were done at RT in capillary quartz tubes, on an ESP300E Bruker X-band machine equipped with a rectangular cavity. The instrumental settings for each set of experiments are specified below.

Binding of Penetratin with Heparin and Heparansulfates

Binding experiments were carried out at room temperature by mixing 50 μl of 0.50 mM MTSL-penetratin dissolved in 50 mM acetate buffer pH 5.5 with increasing volumes of 0.8 mg/ml heparin or 1.6 mg/ml heparansulfates dissolved in the same buffer; variable buffer volumes were added in order to attain a fixed final volume of 100 μl. The final peptide concentration was 0.25 mM in all samples; the heparin concentration ranged between 0 and 0.4 mg/ml; the heparansulfate concentration ranged between 0 and 0.8 mg/ml. All samples were measured within 60 s from mixing. The ESR instrument settings were the following: microwave frequency ~9.4 GHz; microwave power = 2 mW; modulation amplitude = 0.2 G; modulation frequency = 100 kHz. All ESR spectra were subjected to double integration.

Control Binding with Heparin and Heparansulfates

Two distinct controls were carried out: (i) binding of MTSL with heparin and heparansulfates, in the absence of peptide; (ii) competitive binding of labelled and unlabelled penetratin with heparin and heparansulfates.

(i) 50 μl of 0.50 mM MTSL dissolved in 50 mM acetate buffer pH 5.5 was mixed with increasing volumes of 0.8 mg/ml heparin or heparansulfates dissolved in the same buffer, at room temperature; variable buffer volumes were added in order to attain a fixed final volume of 100 μl. The final MTSL concentration was 0.25 mM in all samples; heparin or heparansulfates concentrations ranged between 0 and 0.4 mg/ml.

(ii) 25 μl of 0.25 mM MTSL-penetratin and 25 μl of 0.25 mM unlabelled penetratin dissolved in 50 mM acetate buffer pH 5.5 were mixed with increasing volumes of 0.8 mg/ml heparin or heparansulfates dissolved in the same buffer, at room temperature; variable buffer volumes were added in order to attain a fixed final volume of 100 μl. The final (labelled + unlabelled)-peptide concentration was 0.25 mM in all samples, whereas the final MTSL concentration was 0.125 mM; heparin or heparansulfates concentrations ranged between 0 and 0.4 mg/ml.

All samples were measured within 60 s from mixing. The ESR instrument settings were the following: microwave frequency ~9.4 GHz; microwave power = 2 mW; modulation amplitude = 0.2 G; modulation frequency = 100 kHz. All ESR spectra were subjected to double integration.

Preparation of Phospholipid Vesicles

Vesicles were made of POPG/POPC/PtdSer/cholesterol at different molar ratios. They were prepared by initially dissolving phospholipids (at the desired concentration and in the chosen molar ratio) in chloroform, to ensure the complete mixing of all components, and then removing the solvent by placing the sample under an argon flux for several minutes. The dried lipids were then dissolved in 50 mM acetate buffer

pH 5.5. The ice-cooled dispersion was sonicated for ~30 min, using a heat System Model 350A Sonicator with the microtip at a low output, until the sample became transparent. The sample was then centrifuged in order to remove titanium particles from the microtip. This preparation results in small unilamellar vesicles (Ø < 100 nm) [7,13].

Binding of Penetratin with Lipid Vesicles

Binding experiments were carried out at room temperature by mixing 50 µl of 20 µM MTSL-penetratin dissolved in 50 mM acetate buffer pH 5.5 with increasing volumes of lipid dispersion (prepared as described above); variable buffer volumes were added in order to attain a fixed final volume of 100 µl. The final peptide concentration was 10 µM in all samples; the concentrations of the lipids ranged between 0 and a maximum of 2.0 mM, depending on vesicle formulations. All samples were measured within 60 s from mixing. The ESR instrument settings were the following: microwave frequency ~9.4 GHz; microwave power = 2 mW; modulation amplitude = 3 G; modulation frequency = 100 kHz. All ESR spectra were subjected to double integration.

ESR Spectra Calculations

ESR spectra simulations were carried out by using the EPRsim program (version 4.9), written by J. Strancar [33].

RESULTS

Binding of Penetratin with Heparin and Heparansulfates

A fixed amount of paramagnetically labelled penetratin was incubated with increasing amounts of heparin or heparansulfates. ESR spectra were recorded after each ligand addition, in order to follow the spectral pattern modifications induced by the interaction of penetratin with ligands. Figure 1 shows the ESR spectra of MTSL-penetratin in 50 mM acetate buffer pH 5.5 in the absence and presence of increasing amounts of heparin. The pattern is typical of the MTSL-spin label and is characterized by three lines, due to hyperfine interaction of the unpaired electron with the nuclear spin of a nitrogen atom (I = 1). The slight asymmetry (associated with a reduced intensity of the upfield line) is due to rotational anisotropy. This is related to the fact that the spin-label moiety is bound to the peptide *N*-terminal and thus bears a long peptide queue. As a consequence, the spin-label motion is not equivalent in the three directions of space and a slight broadening of the third spectral line becomes evident with respect to the unconjugated spin-label.

Unexpectedly, no significant change of spectral linewidth was observed during the binding. Nevertheless, a regular and progressive decrease of both the spectral amplitude and the area was observed upon each ligand addition and ended up almost in the disappearance of the ESR trace, as shown in Figure 1. The differential spectral area (i.e. free peptide — peptide/ligand adduct) vs heparin or heparansulfates concentration is reported in Figure 2.

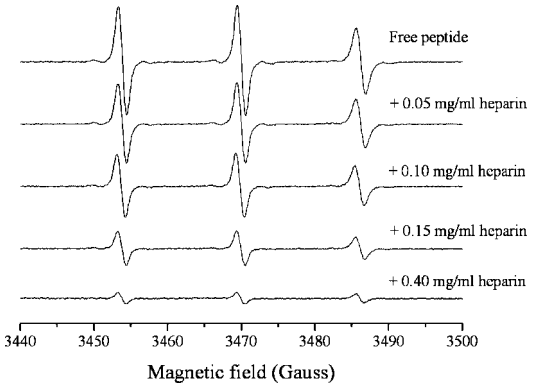

Figure 1 ESR spectra of MTSL-penetratin in the absence and presence of increasing amounts of heparin, recorded at RT. Instrument settings: microwave frequency ~9.4 GHz; microwave power = 2 mW; modulation amplitude = 0.2 G; modulation frequency = 100 kHz.

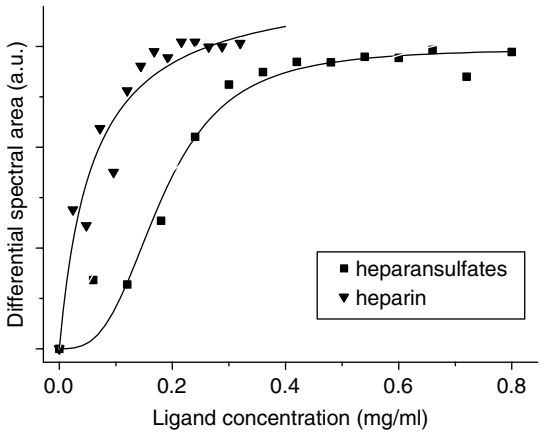

Figure 2 Experimental curves for MTSL-penetratin binding to heparin and heparansulfates; data were fitted with a Hill-type equation (see Table 1).

Two distinct patterns were observed for the two ligands. In order to quantitate these differences, the experimental data were fitted with the following model (a Hill-type equation)

$$y = \frac{\Delta Area_{\max} \times x^n}{Q^n + x^n}$$

where the dissociation constant K_d was replaced by the parameter Q. The assumption behind this model is that the lost intensity of the spectra is proportional to the bound component. The Q parameter can roughly be taken as a partition coefficient and gives an estimate of the relative amounts of free and bound peptide. Figure 2 shows that the interaction of penetratin with heparin and heparansulfates is not equivalent.

The binding curve for heparin is hyperbolic (n = 1.000), whereas the heparansulfates curve is sigmoidal

($n = 3.068$). The Q values relative to each ligand are different as well and suggest that the binding of penetratin to heparin is more favoured with respect to heparansulfates since the Q value in the presence of heparansulfates (0.1812 ± 0.0122 mg/ml) was about ~3-fold that for heparin (0.0590 ± 0.0124 mg/ml). A rough comparison of Q values is allowed by the fact that the average weight of heparin and heparasulfates is similar.

In order to exclude the presence of artifacts due to the interaction of ligands with the spin-label moiety without a real involvement of the peptide, two distinct controls were made: (i) a binding of heparin and heparansulfates with MTSL only (in the absence of peptide); (ii) a competitive binding between the spin-labelled peptide and unlabelled penetratin, in the presence of heparin or heparansulfates.

In the former case, a fixed amount of spin-label MTSL was incubated with increasing amounts of heparin or heparansulfates, in the same experimental conditions described above. No modifications were seen in the three-line ESR spectrum of the MTSL spin-label; this indicates that no interaction between the paramagnetic moiety and the ligands took place.

The competitive binding was carried out incubating equal amounts (0.125 mM) of labelled and unlabelled peptide with increasing amounts of heparin (see Experimental Procedures). The overall peptide concentration was 0.250 mM and the heparin concentration was increased up to 0.8 mg/ml. So, the [peptide]/[heparin] ratio was kept unchanged with respect to the binding experiments previously made. The competitive binding curve (data not shown) was shifted towards higher ligand concentrations with respect to the previous binding experiments. This result is consistent with the presence of competition between the labelled and unlabelled penetratin and also supports the fact that the interaction with heparin involves the whole peptide and not just the spin-label moiety.

Assessment of Penetratin τ_C in the Presence of Heparin and Heparansulfates

Peptide mobility is expressed by the 'correlation time' parameter, τ_C. It was assessed by two distinct methods: (i) the empirical equation based on the linewidth of the middle and upfield lines of the ESR spectrum [9]; (ii) spectral simulation with the software EPRsim [33]. The latter method turned out to be more reliable, whereas the former overestimates τ_C and thus was abandoned. The τ_C value for the free peptide was ~0.12 ns. No significant modification of τ_C values was observed during binding of penetratin with heparin and heparansulfates.

Binding of Penetratin with Phospholipid Vesicles

The interaction of MTSL-penetratin with several phospholipid vesicle formulations was investigated.

The following lipid vesicles were used (molar percentages are specified in brackets):

1. commercial multilamellar liposomes (POPC 52%/POPG 6%/cholesterol 42%)
2. negatively charged unilamellar lipid vesicles (POPG 100%)
3. neutral (zwitterionic) unilamellar lipid vesicles (POPC 100%)
4. mixed unilamellar lipid vesicles (POPG/POPC 50%)
5. negatively charged unilamellar lipid vesicles (PtdSer 100%)

These formulations were chosen by taking into account two factors that are thought to play a role in the internalization mechanism of CPPs: negative charge density and amphiphilicity.

A fixed amount of MTSL-penetratin was incubated with increasing amounts of lipid suspension and the ESR spectra were recorded after a 60 s incubation, at room temperature.

Formulations 1 and 3, that are slightly negatively charged and neutral, respectively, at pH 5.5, did not interact with penetratin at any [lipid]/[peptide] ratio. The ESR spectrum remained unaltered in the whole concentration range and was consistent with the presence of unbound peptide.

Incubation of penetratin with formulations 2, 4 or 5 resulted in a marked line broadening, consistent with the insertion of penetratin inside the lipid bilayer (Figure 3). Spectral simulation shows that a slow-motion component comes out in the ESR spectrum, that is associated with high τ_C values and indicates that a striking decrease of peptide mobility is occurring.

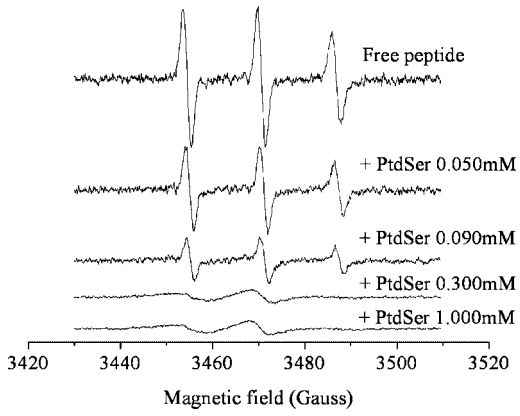

Figure 3 ESR spectra of MTSL-penetratin in the absence and presence of increasing amount of 100% mol PtdSer vesicles, recorded at RT. Instrument settings: microwave frequency ~9.4 GHz; microwave power = 2 mW; modulation amplitude = 3 G; modulation frequency = 100 kHz.

The most relevant experimental finding concerning the interaction of penetratin with negatively charged phospholipid vesicles is the existence of two concentration-dependent regimes. At a low [lipid]/[peptide] ratio, the ESR spectra follow the same trend as that observed with heparin and heparansulfates: the ESR pattern does not change, but its amplitude, as well as the spectral area, decreases. Once a critical threshold is reached (i.e. at a higher lipid concentration), the spectral pattern abruptly changes and broadened lines appear (Figure 3).

This behaviour is clearly evident in Figure 4 that shows the area of ESR spectra vs the total lipid concentration when MTSL-penetratin is incubated with POPG 100% unilamellar vesicles. In the low lipid regime, the area slowly decreases and reaches a minimum point; beyond this point (i.e. in the high lipid regime), spectrum linewidths broaden and the area increases, although it never returns to the initial value. A similar trend was found with lipid vesicle formulations 4 and 5 (data not shown). This is the first time that such a concentration-dependent change of the ESR pattern has been reported in the literature and represents a new finding with respect to previous ESR investigations on this topic. No variation of the ESR spectral area of labelled penetratin in the presence of phospholipid vesicles had ever been pointed out and the only reported spectral change was linewidth broadening, following insertion of penetratin in the lipid bilayer.

Figures 5A and B report the τ_C values calculated from ESR spectra of penetratin, in the presence of either 100% POPG or 50% POPG/50% POPC vesicles, at increasing [lipid]/[peptide] ratios. The spectra turned out to be the sum of two components: an isotropic one, associated with low τ_C values (from 0.12 ns up to 1 ns); and an anisotropic one, associated with high τ_C values (2–3 ns). The relative % of such components varies all over the concentration range, as shown in Figure 6.

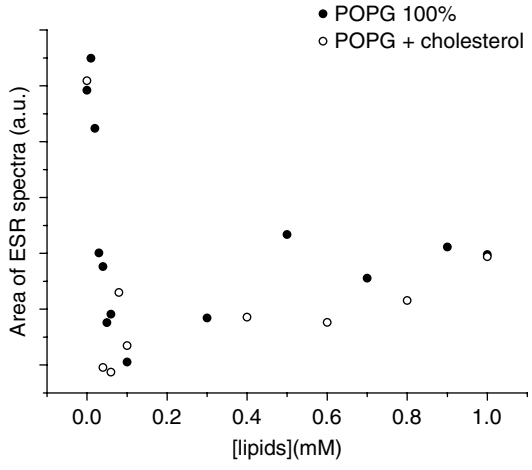

Figure 4 Area of the ESR spectra recorded during binding of MTSL-penetratin with (100% mol POPG) vesicles or (60 mol% POPG/40 mol% cholesterol) vesicles.

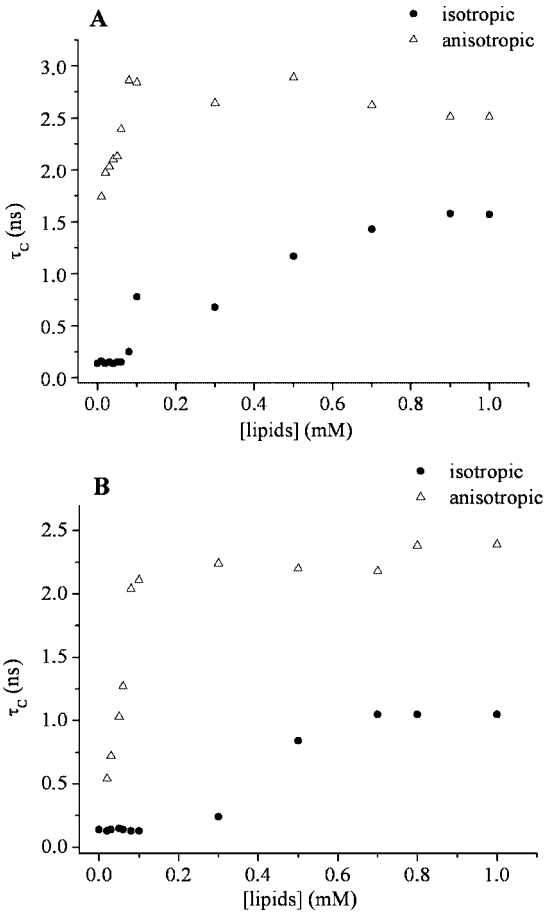

Figure 5 (A) τ_C values associated with the isotropic and anisotropic components of the ESR spectrum relative to binding of MTSL-penetratin with (100% mol POPG) vesicles; (B) τ_C values associated with the isotropic and anisotropic components of the ESR spectrum relative to binding of MTSL-penetratin with (50% mol POPG/50% mol POPC) vesicles.

At low lipid concentrations, the isotropic component prevails. But, as soon as the lipid concentration increases, its contribution becomes smaller and the spectrum is mainly described by the anisotropic feature, that reaches a plateau at a high [lipid]/[peptide] ratio. The high τ_C values associated with the anisotropic component indicate low mobility of the paramagnetic probe, consistent with peptide entrapment into the lipid bilayer. Conversely, τ_C values associated with the isotropic part of the spectrum, that prevails at low lipid concentration, are comparable to those typical of free peptide (0.12 ns).

The intersection point of the curves reported in Figure 6 represents a critical value of the [lipid]/[peptide] ratio, that is to say a threshold. Starting from this point, the anisotropic component prevails over the isotropic one and linewidth broadening becomes evident in the ESR pattern. The threshold is dependent on the composition of lipid vesicles: each formulation turned out to have a specific [lipid]/[peptide] threshold

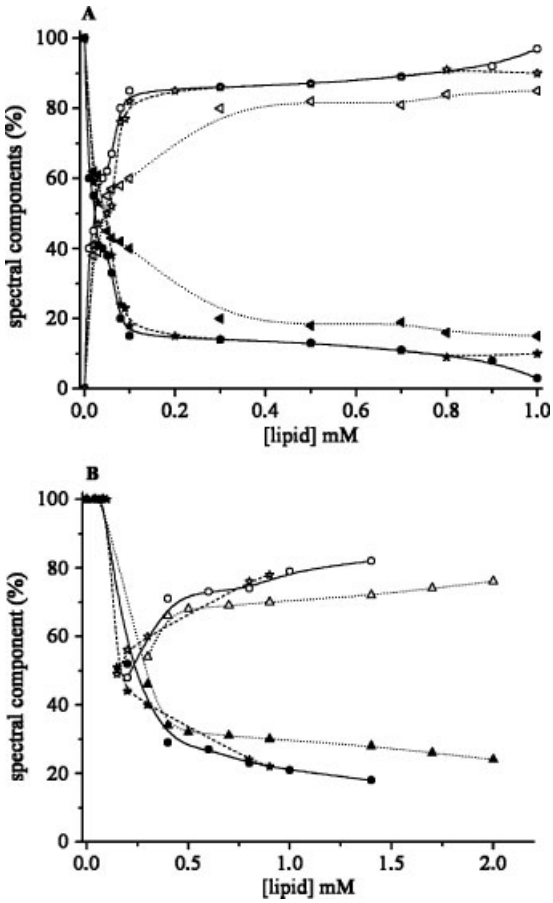

Figure 6 (A) relative % of the isotropic and anisotropic components of ESR spectra recorded during binding of MTSL-penetratin with cholesterol-lacking vesicles. (B) relative % of the isotropic and anisotropic components of ESR spectra recorded during binding of MTSL-penetratin with cholesterol-containing vesicles. Legend: POPG 100% (closed circles = isotropic; open circles = anisotropic); POPC50%/ POPG50% (closed triangles = isotropic; open triangles = anisotropic); PtdSer 100% (closed stars = isotropic; open stars = anisotropic).

value, as shown in Table 1. It is worth noting that it roughly corresponds to the minimum in the (area vs concentration) plot.

Effect of Cholesterol

The presence of cholesterol in the lipid bilayer has been reported to discourage penetratin insertion into the membrane [1]. In order to check the effect of cholesterol, three new formulations of phospholipid vesicles were tested. These are analogous to formulations 2, 4 and 5, but contain 40 mol% cholesterol. They have been called 2′, 4′ and 5′ and underwent the same treatments as the cholesterol-lacking vesicles.

The results obtained on the cholesterol-containing formulations are shown in Table 1 and Figures 4 and 6B. A comparison between the cholesterol-containing and cholesterol-lacking vesicles shows that:

Table 1 [Total lipids]/[Penetratin] Threshold Values associated with the Insertion of Penetratin into Lipid Vesicles

Vesicle formulation (in mol%) *(lipid concentration range used in the binding experiment)*	Threshold
POPG 100% *(0–1 mM)*	8
60% POPG + 40% cholesterol *(0.0–1.4 mM)*	20
50% POPG + 50% POPC *(0–1 mM)*	30
30% POPG + 30% POPC + 40% cholesterol *(0.0–2.0 mM)*	50
PtdSer 100% *(0–1 mM)*	10
60% PtdSer + 40% cholesterol *(0.0–0.09 mM)*	15

(i) the addition of cholesterol shifts the [lipid]/[peptide] threshold towards higher values: the effect is more evident in formulations 2′ and 4′ with respect to 5′; (ii) whenever cholesterol is present, a single isotropic component is found in the ESR spectrum at a low [lipid]/[peptide] ratio; the anisotropic component of the ESR spectrum appears much later in the binding with respect to the cholesterol-lacking vesicles, that is to say at higher ligand concentrations (see Figures 6A and B); (iii) the τ_C values associated with the anisotropic component in formulation 2′ are similar to those obtained in the absence of cholesterol (about 3.0 ns); conversely, formulations 4′ and 5′ were characterized by significantly lower values (1.5–2.0 ns) in similar conditions (data not shown).

DISCUSSION

The Interaction of Penetratin with Heparin and Heparansulfates

The decrease of ESR signal amplitude (and area) observed during binding of heparin and heparansulfates to penetratin is due to a decrease of the number of spins in the sample. This can be explained by spin-spin coupling between peptide *N*-terminals which, in turn, may be a consequence of peptide aggregation.

This experimental finding agrees with the literature available on this topic. Peptide–peptide interactions have already been reported to take place on the surface of lipid vesicles [6]. Similarly, penetratin has been found to induce aggregation of negatively charged lipid vesicles [5], witnessed by an increase of turbidity. A conformational transition of penetratin from α-helix to β-sheet, associated with aggregation, has been observed also [7,5,10,11].

The biological significance of these data comes from the evidence that heparin and heparansulfate proteoglycans are known to be involved in peptide internalization [14,16,34]. According to Tiagy *et al.* [16], they can act as receptors for extracellular TAT uptake.

Their interaction with TAT is claimed to be specific and determined by size, saccharides composition and extent and distribution of sulfation of the GAG backbone. Yanagishita *et al.* [31] report that their ability to interact with other macromolecules is largely determined by their highly negatively charged chains.

Suzuki *et al.* [34] suggest that cell-surface sulfated polysaccharides may contribute to the internalization of arginine-rich peptides (as penetratin) by interacting electrostatically with them. In particular, these polysaccharides are thought to promote peptide concentration on the cell surface. Similarly, Mai *et al.* [14] show that internalization of arginine-rich peptides is reduced in heparansulfates- or glycosaminoglycan-deficient cell lines, although it can be enhanced by addition of dextran sulfate. A number of authors [14,15,19,35–37] assert the importance of electrostatic interactions between positively charged amino acid residues (such as Arg and Lys) and negatively charged sulfate groups (such as those found in heparin and heparansulfates) in peptide internalization.

When examined within this frame, our data suggest that electrostatic interaction of the Arg and Lys residues of penetratin with sulfate groups found on the sugar moieties are established during binding experiments. Heparin and heparansulfates seem to induce peptide molecules to concentrate on their molecular surface and promote spin-coupling as a consequence of peptide aggregation. Significantly, some turbidity of the acqueous solution was noticed during the binding experiments. Although both heparin and heparansulfates interact with penetratin, their binding mode is not equivalent, as shown by the different pattern of the binding curve as well as by the distinct Q values. Although this parameter has a semi-quantitative meaning, its values suggest that the affinity of penetratin for heparin is higher than for heparansulfates. This is consistent with the higher sulfate content found in heparin with respect to heparansulfates, which implies a higher negative charge density that, in turn, plays a key role in the mechanism of CPP internalization [1,13,14,29,34,35].

The almost invariance of τ_C values during binding is not surprising: in fact, the ESR signal observed refers to unbound peptide, since the bound peptide aggregates and becomes ESR-silent. This also explains why slow-motion structures (i.e. large linewidth spectra) are not detected by ESR. The ESR spectra suggest that the amount of free peptide decreases progressively as soon as binding saturation is approached. Finally, control experiments made in the absence of penetratin and competitive binding unequivocally show that all the observed spectral changes are not experimental artifacts due to spin-label interactions, but they are strictly dependent on peptide concentration.

The Interaction of Penetratin with Phospholipid Vesicles

Penetratin binding to phospholipid vesicles is more complex than the previous one. It was shown that no interaction took place in the presence of neutral or slightly negatively charged lipids. Conversely, penetratin interacted strongly with highly negatively charged lipids and the interaction features depend on charge density, that is a consequence of the chemical composition of lipid vesicles.

Another crucial factor seems to be the size and number of lipid layers found in lipid vesicles: commercial multilamellar liposomes ($\varnothing > 100$ nm) do not interact at all with penetratin, even if they bear a slightly negative charge density. Conversely, negatively charged unilamellar vesicles ($\varnothing < 100$ nm) incorporate penetratin into the lipid bilayer, as witnessed by the appearance of broadened ESR lines (see Figure 3).

The main feature is the two-regime behaviour, that is dependent on lipid concentration and was found with formulations 2, 4 and 5, as well as 2′, 4′ and 5′.

The low-lipid regime is somehow analogous to what is observed during binding of penetratin with heparin and heparansulfates. It is characterized by the presence of magnetic coupling phenomena, associated with peptide aggregation, resulting in a progressive decrease of the spectral area. This is certainly mediated by lipid vesicles and cannot be observed in their absence, even at high peptide concentrations.

The high lipid regime is characterized by an abrupt broadening of the ESR lines, as well as by an increase of spectral amplitude. This indicates that peptide enters the lipid bilayer and that partial disaggregation occurs (since the spectral amplitude never goes back to the original values and part of the spin is irreversibly lost).

The passage from the first to the second step is quite abrupt and occurs as soon as the [lipid]/[peptide] ratio reaches a critical value, that is typical for each vesicle formulation. Below this threshold, i.e. in the low lipid regime, most of the peptide is found free in solution (its amount decreases as soon as the threshold is approached), while a smaller portion is aggregated on the vesicle and is non-visible by ESR. This explains why the ESR spectrum is mainly described by an isotropic component, associated with low τ_C values. Above the threshold, i.e. in the high lipid regime, the spectrum is mainly described by an anisotropic component, associated with high τ_C values (~3 ns) and broad lines; simultaneously, the spectral amplitude starts to increase. This suggests that a relevant fraction of bound peptide is now inside the membrane and that disaggregation is occurring. This also indicates that the *N*-terminal side of penetratin (which bears the spin-label moiety) must be localized inside the bilayer. Spin–spin interaction can be excluded as the origin of the line broadening, since it never occurs at high peptide concentration in the absence

of lipids; moreover, a pure peptide aggregate is not likely to be associated with such high τ_C values. If the [lipid]/[peptide] threshold is adopted as a reference, it is noticed that vesicles made of 100% POPG seem to be better ligands than those made of equimolar amounts of POPG and POPC, since they are characterized by a lower threshold value. Even vesicles made of 100% PtdSer, although characterized by a high charge density, are less efficient than pure POPG in promoting the insertion of penetratin into the membrane.

These data substantially agree with the literature available on the interaction between CPPs and liposomes, in the frame of peptide internalization studies. According to most authors, a critical step of the internalization process consists of the electrostatic interaction between positively charged peptide residues (e.g. Lys and Arg) and the negatively charged head of phospholipids. More specifically, the guanidino moiety has been proposed to form a hydrogen bonding structure with phospholipids in the lipid bilayer [12,14,27,29,30,35–37]. It is also interesting that the [lipid]/[peptide] threshold value of 8, observed in the presence of 100% POPG vesicles, matches exactly the result reported by several research groups as an ideal value for peptide internalization in lipid vesicles made either of 100% POPG or 100% PtdSer [12,29,35,36].

Our experimental findings are fairly consistent with those published by Magzoub *et al.* [10], according to which penetratin lies perpendicular to the bilayer normal in POPG/POPC vesicles. No evidence has been found for penetratin to be released into the aqueous phase inside the lipid vesicles: once pAntp penetrates inside the lipid bilayer, it sticks to it. A similar finding has been reported by Binder *et al.* [30], who claim that penetratin crosses the lipid membrane and lays on the inner surface of the vesicles bilayer, whereas pAntp internalization is known to occur in cells.

Cholesterol perturbs peptide insertion into the lipid membrane: this is witnessed by the general increase of the [lipid]/[peptide] threshold value observed in cholesterol-containing vesicles with respect to the analogous cholesterol-lacking formulations, as reported in Table 1, and by the different relative distribution of the isotropic and anisotropic component of the ESR spectra shown in Figure 6A and B. Our result is in agreement with those reported by Christiaens *et al.* [1].

The perturbing effect of cholesterol is slightly less with POPG than with mixed POPG/POPC or PtdSer vesicles. This is highlighted by the τ_C values associated with the anisotropic component, when binding saturation is attained: ~3 ns with POPG/cholesterol vs 1.5–2.0 ns with POPG/POPC/cholesterol or PtdSer/cholesterol. This means that peptide inclusion into the membrane is not complete in the latter case and is likely related to the increased membrane rigidity brought about by cholesterol itself.

Our interpretation of the overall results obtained in the presence of heparin (or heparansulfates) and phospholipids vesicles are consistent with the biphasic model first proposed by Prochiantz [8,24–27,29], that implies two simple steps: (i) peptide interaction with negatively charged sugars induces penetratin to concentrate on the cell surface; (ii) peptide interacts with lipid polar heads and subsequently enters the membrane.

It is also worth noting the substantial agreement of our findings with those reported by Binder *et al.* [30]. They ascribe crucial importance to electrostatic interactions between peptide and lipids, since they are effective in perturbing the transmembrane electric field and in promoting peptide aggregation onto the vesicle surface. Moreover, they also notice that a [lipid]/[peptide] threshold has to be attained in order to allow peptide insertion in the membrane. Its value is related to the negative charge density on the membrane surface.

In conclusion:

(i) Both heparin and heparansulfates interact with spin-labelled penetratin, although their interaction is not equivalent, as shown by the ESR technique.
(ii) These polysaccharides are likely to play a role in the initial phase of the internalization process by promoting peptide aggregation and concentration on their molecular surface.
(iii) The behaviour of penetratin in the presence of distinct phospholipid vesicle formulations confirms that surface charge density is a crucial factor in peptide–lipid interactions.
(iv) Penetratin enters negatively charged lipid bilayers, as shown by the 3 ns τ_C value calculated from ESR spectra.
(v) The presence of cholesterol is unfavourable to peptide insertion into the lipid bilayer, likely due to increased membrane rigidity.
(vi) The interaction of penetratin with lipid vesicles strongly depends on lipid concentration: at a low [lipid] regime, the peptide associates with the polar heads of phospholipids on the membrane surface and aggregates on it; as soon as the lipid concentration increases and a threshold value of the [lipid]/[peptide] ratio is attained, penetratin enters the lipid bilayer and partially disaggregates. No evidence has been found for penetratin to reach the aqueous phase inside the lipid vesicles.
(vii) The concentration-dependent behaviour observed in this study agrees with analogous experimental findings by other groups [8,14,24–27,29,30].
(viii) This work shows that ESR spectroscopy can provide extremely specific information on the interaction of carrier-peptides with cell membrane components, at a molecular level.

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