Penetratin-induced aggregation and subsequent dissociation of negatively charged phospholipid vesicles

Daniel Persson[1], Per E.G. Thorén[1], Bengt Nordén*

Department of Physical Chemistry, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden

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Abstract The interaction of the cellular delivery vector penetratin with a model system consisting of negatively charged phospholipid vesicles has been studied. Above a certain peptide to lipid molar ratio, the cationic oligopeptide induces vesicle aggregation. Interestingly, the aggregation is followed by spontaneous disaggregation, which may be related to membrane translocation of the peptide. Circular dichroism (CD) measurements indicate a conformational transition, from α-helix to antiparallel β-pleated sheet, which is simultaneous with the aggregation process. The potential influence of spectroscopic artifacts on CD data due to the drastically increased turbidity during aggregation is discussed. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antennapedia homeodomain; Penetratin; Vesicle aggregation; Circular dichroism; Artifact; Turbidity

1. Introduction

Penetratin, also denoted pAntp, is a 16-mer peptide derived from the homeodomain of the *Drosophila* transcription factor Antennapedia [1]. It belongs to a class of peptides with a remarkable ability to enter cells through an apparently non-endocytotic and receptor- and transporter-independent pathway, even when linked to molecular cargos such as oligonucleotides and proteins [2–4]. The high efficiency of internalization makes these peptides particularly interesting for e.g. antisense/antigen applications. The membrane translocation mechanism of penetratin remains a puzzle, even though a number of studies, in cells [5–7] as well as in lipid model systems [8–13], have contributed to the current knowledge of the properties of the peptide.

In the present work we report that penetratin, above a certain peptide to lipid (P:L) molar ratio, induces aggregation of negatively charged phospholipid vesicles, followed by spontaneous disaggregation. Peptide circular dichroism (CD) data indicate that vesicle aggregation is accompanied by a conformational transition from α-helix to antiparallel β-pleated sheet. In this context, the possible occurrence of spectroscopic artifacts due to the drastically increased turbidity during aggregation is discussed.

The stability of a dispersion of charged vesicles is mainly governed by three types of forces: electrostatic repulsion, van der Waals attraction and hydration forces. The former two are long-range forces whereas the hydration forces, which can be ascribed to the hydration of the lipid headgroups, are significant at shorter intermembrane distances (below 20–30 Å). There are several ways to manipulate the contributions from these interactions. The electrostatic forces are affected by the surface charge density of the vesicles and the electrolyte concentration, while the van der Waals interactions can be modulated by changing e.g. the vesicle size. The magnitude of the hydration forces is intimately related to the energy required to remove water from the membrane surface [14]. It has been shown that several polyvalent cations, apart from altering the surface charge density upon adsorption to the membrane, dehydrate the lipid headgroups and thereby induce aggregation of phospholipid vesicles [14,15]. Since penetratin carries seven formal positive charges, the destabilizing effect on a dispersion of negatively charged vesicles reported here is not surprising. The spontaneous dissociation of the aggregates, on the other hand, is an unexpected dissipative process that might reflect the redistribution of peptide by membrane translocation.

2. Materials and methods

2.1. Chemicals

Standard Fmoc-protected amino acids were obtained from Nova Biochem (Arg, Lys, Met, Trp, Phe), Alexis Corporation (Gln, Asn) and Perseptive Biosystems (Ile).

1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma. Ethylenediaminetetraacetic acid (EDTA) (titriplex III) was obtained from Merck. Deionized water from a Milli-Q system (Millipore) was used. Penetratin (Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) was synthesized by Fmoc solid-phase synthesis as described elsewhere [10].

2.2. Preparation of large unilamellar vesicles (LUVs)

A dry lipid film of DOPG was prepared from a chloroform solution by removing the solvent on a rotary evaporator. The lipid film was placed in high vacuum for 2 h to remove trace amounts of chloroform. Vesicles were prepared by dispersion of the lipid in buffer by vigorous vortexing. The multilamellar vesicles were subjected to five freeze–thaw cycles before extrusion 21 times through two 100 nm

*Corresponding author. Fax: (46)-31-7723858.
E-mail addresses: danielp@phc.chalmers.se (D. Persson), thoren@phc.chalmers.se (P.E.G. Thorén), norden@phc.chalmers.se (B. Nordén).

[1] Both authors contributed equally to this work.

Abbreviations: CD, circular dichroism; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); LUV, large unilamellar vesicle; DLS, dynamic light scattering

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polycarbonate filters on a LiposoFast-Pneumatic extruder (Avestin) to obtain LUVs. The buffer used for liposome preparation was either 10 mM HEPES, 0.1 M NaCl, 5.3 mM NaOH and 1 mM EDTA (pH 7.4) or 10 mM sodium phosphate buffer (pH 7.4).

2.3. Measurements of vesicle aggregation and dissociation

Turbidity or static light scattering was employed to continuously monitor aggregation and dissociation of liposomes. Due to its larger reliability and superior applicability for measuring particle size growth [16], turbidity was preferentially used in this work. Turbidity measurements were performed on a Cary 4B UV-Vis spectrometer at 436 nm. A Spex Fluorolog τ-3 spectrofluorometer (JY Horiba) was used for static light scattering experiments. The excitation and emission monochromators were both set to 600 nm and the bandpasses to 0.5 and 2.0 nm, respectively. All measurements were carried out at room temperature in a 1 cm quartz cell with a sample volume of 3 ml. Liposomes and peptide were added from stock solutions with concentrations of 5 mM and 100 μM, respectively. Dynamic light scattering (DLS) experiments were performed using a Malvern Instrument Series 7032 Multi-8 correlator and a PCS100 spectrometer (Malvern Instruments).

2.4. CD measurements

CD was measured on a Jasco J-720 spectropolarimeter using a 1 cm quartz cell. All spectra were taken between 190 and 260 nm and corrected for background contributions. Results are expressed as mean residue ellipticities $[\theta]_{MR}$ (° cm^2/dmol). Reference CD spectra were taken from Perczel et al. [17]. Secondary structure evaluation was performed by least-square projection of the acquired spectra (between 195 nm and 240 nm) on α-helix, antiparallel β-pleated sheet and random coil reference spectra using Matlab (The MathWorks Inc.).

3. Results

3.1. Aggregation and dissociation of DOPG LUVs caused by penetratin

Fig. 1A shows the effect of penetratin addition on the turbidity of a suspension of DOPG liposomes. The liposomes, 100 nm in diameter, were mixed with peptide at a defined P:L molar ratio and the optical density at 436 nm was measured with time. At low P:L ratios (up to 1:15), no significant change in turbidity was observed. However, at a certain penetratin concentration, corresponding to a P:L ratio of 1:13.6 (trace b), liposome aggregation causes a rapid increase in turbidity. DLS experiments confirmed that large aggregates were formed. Due to the difficulties in accurately determining the diffusion autocorrelation function for DLS measured on systems undergoing rapid changes in particle size, the numbers from the data evaluation should be considered merely as rough estimates of the actual sizes of the aggregates. Nevertheless, the time dependence of the calculated mean size of the aggregates is consistent with that of the turbidity. For example, at a ratio of 1:12.5 (cf. Fig. 1A, trace c), the average size goes from 100 nm before addition of peptide to about 500 nm after 5 min and has its largest value (∼1 μm) after 25 min, when the optical density has reached its maximum of about 0.04. These numbers agree well with observations from studies of reversible, Be^{2+}-induced aggregation of PC LUVs [18]. Interestingly, the aggregation is followed by dissociation, which is manifested by a slow reduction of the turbidity. The kinetics of the dissociation are strongly dependent on the P:L ratio, but in all cases shown in Fig. 1A, the optical density returns to the initial level. This was accordingly also the case for a P:L ratio of 1:7.5 (trace d), although it required more than 12 h. The fact that the initial and the final optical densities have the same value indicates complete dissociation of the aggregates. This conclusion was also supported by static light

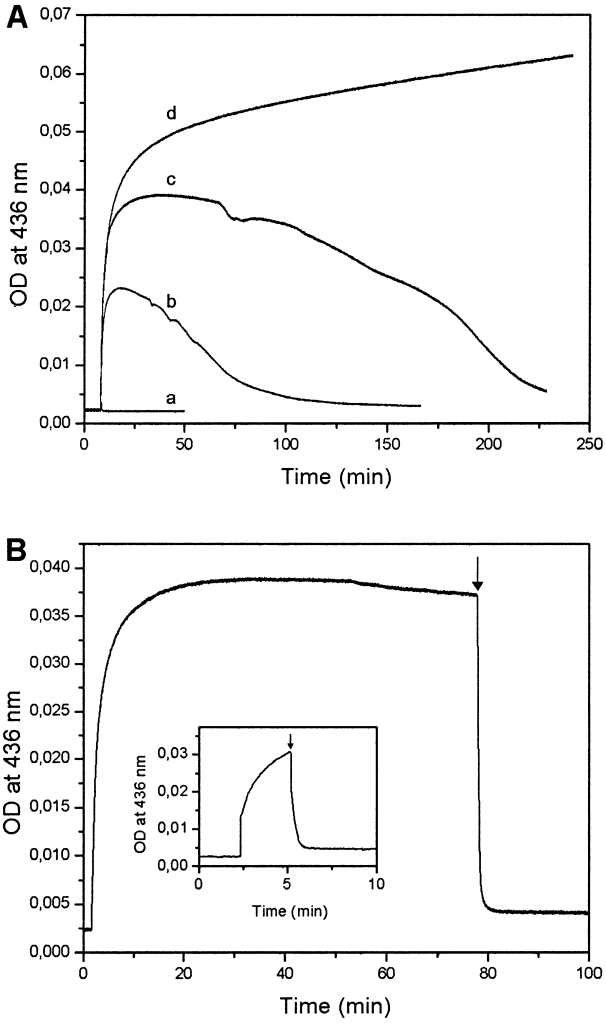

Fig. 1. A: Time course of the optical density at 436 nm showing aggregation and dissociation of DOPG LUVs (100 nm in diameter) induced by penetratin. After 10 min, peptide stock solution was added to the vesicles (25 μM DOPG) in HEPES buffer, resulting in a P:L molar ratio of 1:15 (trace a), 1:13.6 (b), 1:12.5 (c) or 1:7.5 (d). B: Effect of addition of liposome stock solution to an aggregated sample. First, vesicle aggregation was induced by peptide addition (P:L ratio 1:12.5). Rapid disaggregation was then accomplished by addition of a second set of liposomes (arrow), resulting in a final P:L ratio of 1:25. The experiment could be repeated with liposome addition at different time points. The inset shows dissociation induced in the early stages of the aggregation process.

scattering measurements, where the intensity of scattered light after dissociation exactly matches that before peptide addition (data not shown). DLS experiments definitively confirmed the restitution of non-aggregated 100 nm liposomes. The aggregation/dissociation process is not dependent on the order of peptide and liposome addition. When adding liposome stock solution to a peptide sample instead of placing peptide stock solution in a liposome dispersion, the result is exactly the same (data not shown).

Two supplementary experiments were carried out, providing additional information. After dissociation, further peptide addition causes reaggregation of the liposomes, again followed by dissociation. During this process, the optical density follows a similar pattern as that after the first peptide addition. Furthermore, complete disaggregation can be rapidly

accomplished by addition of a second set of liposomes resulting in a P:L ratio below 1:13.6 (Fig. 1B). This can be done at any time point during the course of the aggregation/dissociation process (Fig. 1B, inset). If the lipid concentration is doubled in this addition, the resulting optical density after dissociation is exactly twice that of the sample before aggregation, in agreement with dissociation leading to non-aggregated 100 nm liposomes. These results show that the peptide molecules are readily redistributed between the aggregates and the vesicles added afterwards.

The results presented in Fig. 1 were obtained using a standard HEPES buffer with 0.1 M NaCl, but a similar behavior was observed with liposomes suspended in a 1 mM phosphate buffer (pH 7.4). One difference was that a higher P:L ratio (1:10) was required for aggregation. A representative example of aggregation and dissociation in phosphate buffer is shown in Fig. 2A. As can be seen, the overall shape of the aggregation/dissociation curve differs from those obtained using HEPES buffer.

3.2. Conformation of penetratin

In order to avoid extensive absorption in the far UV region, 1 mM phosphate buffer was used for CD experiments. In buffer, the peptide was found to adopt a random coil conformation (data not shown), in agreement with previous findings [8,12]. At low P:L ratios, where no aggregation occurs, the membrane-bound peptide has a CD spectrum characteristic of an α-helix (data not shown).

When using a P:L ratio high enough to induce aggregation, the shape of the CD spectrum is time-dependent and coupled to the aggregation and dissociation of the liposomes. In Fig. 2A (top trace), the change in optical density with time at a P:L ratio of 1:8.3 is shown. The time required for the entire process may vary slightly between individual samples, but the shape of the curve is the same. After peptide addition, we measured five consecutive CD spectra, each spectrum being an average of 20 scans recorded during a total of 30 min. In Fig. 2, these spectra are referred to as I–V. Spectrum I in Fig. 2B, taken during the first 30 min, deviates significantly from that of an α-helix. Instead, it shows the characteristics of an antiparallel β-pleated sheet spectrum. The relative contributions from antiparallel β-sheet, random coil and α-helix indicated by secondary structure evaluation were 72%, 19% and 9%, respectively. The second spectrum is similar to the first, although slightly shifted towards an α-helix spectrum (62% antiparallel β-sheet, 13% random coil, 25% α-helix). Note that these spectra were recorded when the liposomes were assembled in large aggregates, which could complicate the evaluation due to spectroscopic artifacts such as absorption statistics and depolarization by light scattering. There are, however, a number of arguments that the observed shift from α-helix- to β-sheet-like spectra is not an artifact (see Section 4). The remaining three spectra (III–V) are all α-helical and identical to those obtained in the absence of aggregation (at low P:L ratios). No β-sheet component was found in these spectra.

For a closer examination of the CD kinetics, the CD at 209 nm was followed with time (Fig. 2A, lower trace). At this wavelength, there is a pronounced difference between spectrum I and spectrum V, while the photomultiplier high tension voltage and thereby also the noise level remain low. The CD signal follows the expected pattern, rising rapidly from negative values to slightly above zero during the fast aggregation, peaking at 5000° cm^2/dmol and then decaying to a level corresponding to the signal at 209 nm in spectra III–V. Comparison with the optical density plot in Fig. 2A reveals a strong correlation between the aggregation/dissociation process, as manifested by the turbidity, and the changes in the CD signal. The fact that spectra III–V overlap thus makes it reasonable to assume that the liposomes in this sample are no longer aggregated as the acquisition of spectrum III is initiated.

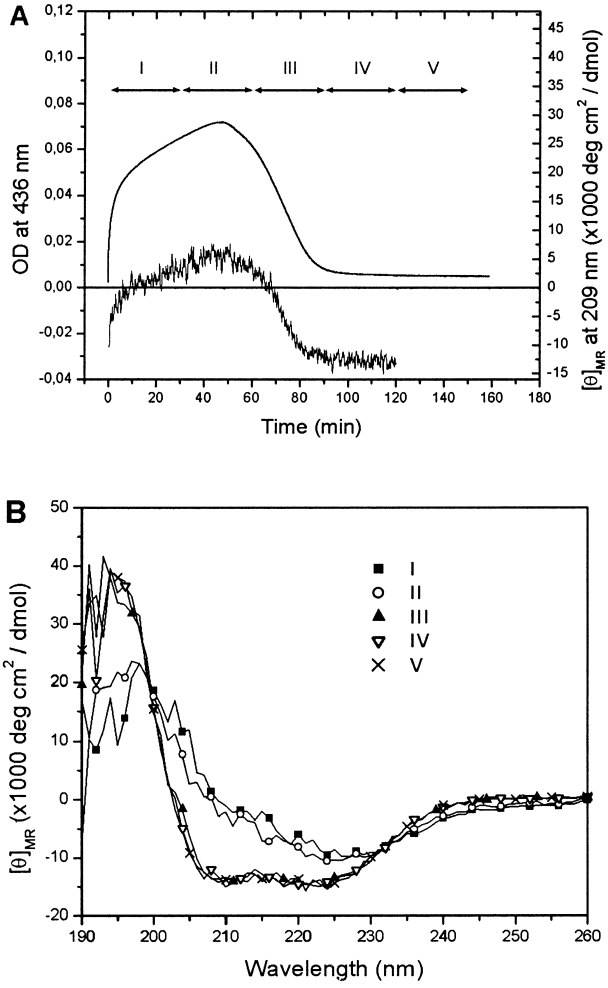

Fig. 2. A: Optical density (OD, upper trace) at 436 nm and mean residue ellipticity, $[\theta]_{MR}$, at 209 nm (lower trace) as a function of time after addition of peptide stock solution to DOPG LUVs (25 μM) in phosphate buffer. The P:L molar ratio was 1:8.3. The arrows (I–V) indicate the acquisition periods of the CD spectra presented in B. B: Consecutive CD spectra collected after peptide addition to a liposome sample (P:L ratio 1:8.3). Each spectrum is an average of 20 scans recorded during a total of 30 min.

A control experiment was performed to assess the possible influence from light scattering on the CD spectrum of the membrane-bound penetratin. It is known that light scattering may cause depolarization of polarized light, leading to perturbations in the CD signal. A quartz cell containing penetratin (1 μM) bound to liposomes (12.5 μM DOPG) in phosphate buffer was placed in the instrument, preceded in the light path by another cell containing liposomes (12.5 μM DOPG) only. The peptide is α-helical at this P:L ratio. After measuring the CD spectrum of the peptide in this setup, the liposomes in the peptide-free cell were aggregated/fused by addition of $CaCl_2$

to a final concentration of 3 mM. A stable turbidity matching the peak value of the optical density in Fig. 2A was obtained after 0.5 h and a second CD spectrum was acquired. It was found to be identical to the first one, indicating that no significant depolarization occurred.

In earlier CD studies, penetratin has been found to adopt either an α-helical conformation [12] or an antiparallel β-sheet structure [11] when bound to negatively charged phospholipid vesicles. The results presented here might explain the differences (see Section 4).

4. Discussion

The most important finding in this work is that penetratin causes aggregation of DOPG vesicles followed by spontaneous dissociation. Such a process has, to our knowledge, never been observed before.

The aggregation observed at high P:L ratios may involve several different effects caused by penetratin binding to the vesicles. The most obvious effect is the influence on the electrostatics associated with the introduction of a polycation (+7) in the headgroup region of the negatively charged lipid membrane. This reduces the surface charge density of the liposomes and thus the electrostatic repulsion stabilizing the vesicle dispersion. It is furthermore possible that the observed formation of β-sheet structures leads to alternating regions of positive and negative surface charge due to an uneven distribution of peptide molecules. If so, attractive electrostatic forces could arise between adjacent bilayers. A second stabilizing factor is the hydration shell surrounding the liposomes, giving rise to repulsive hydration forces between approaching membrane surfaces. It is plausible that extensive binding of the peptide interferes with the hydration shell, rendering the vesicles more prone to aggregation. Protein-induced dehydration has previously been reported for annexin V interacting with PS vesicles [19]. Yet another contribution might stem from a peptide-induced stiffening of the membrane at elevated P:L ratios. This would result in suppression of the bilayer undulations, which play a significant role for the repulsive interactions of lipid membranes in the liquid crystalline state [20]. Since the temperature chosen in these experiments (25°C) is well above the phase transition temperature of DOPG, the potential influence of the peptide on the bilayer undulations should be considered. Clearly, quantification of the relative contributions from the effects of penetratin on the intervesicular interactions (electrostatic forces, hydration forces, undulation forces, etc.) is not possible. It is, thus, difficult to make a direct interpretation of the critical P:L ratio, above which aggregation occurs. However, it is noteworthy that the critical P:L ratio is clearly dependent on the buffer used (1:13.6 in 10 mM HEPES with 100 mM NaCl and 1:10 in 1 mM phosphate). This most likely reflects the importance of the electrostatic contribution, which is expected to differ between these systems.

There are numerous examples in the literature of peptides causing aggregation of liposomes. In most cases reported, however, aggregation is followed by vesicle fusion [21–23]. Our results clearly indicate that penetratin does not induce fusion of DOPG vesicles. Instead, an unexpected dissociation of the aggregated vesicles occurs. There are a number of possible explanations for this phenomenon. One trivial cause would be locally high penetratin concentrations upon addition of peptide stock solution, resulting in unevenly populated liposomes. As a consequence, the liposomes with little or no peptide bound and those with high peptide surface coverage might be prone to aggregation due to e.g. charge reversal. Redistribution of penetratin would level out the differences in the amount of associated peptide between the liposomes, abolishing this origin of liposome aggregation. This explanation is, however, contradicted by the fact that the order of peptide and liposome addition to the sample does not matter. The occurrence of a critical P:L ratio for aggregation is also inconsistent with this reasoning. Furthermore, since the dissociation occurs on a long timescale, the intervesicular redistribution of peptide would have to be slow in order to explain the experimental results. The rapid dissociation when a second set of liposomes is added to the aggregates (Fig. 1B) demonstrates that this is not the case. The possibility that the liposome disaggregation is caused by degradation of peptide due to some impurity in the samples can also be ruled out, since the CD signal as well as the tryptophan fluorescence of the peptide are constant with time in the absence of aggregates (data not shown).

A third explanation would involve membrane charge reversal in combination with slow peptide binding. Although penetratin at low P:L ratios associates rapidly with lipid membranes (unpublished results), it is plausible that highly elevated peptide concentrations at the membrane surface and/or aggregation of the vesicles slow down the peptide binding kinetics to the timescale of these experiments. As the association of peptide proceeds, the net charge of the lipid membranes would become less negative and at some point, charge reversal would occur. At a high enough positive charge, electrostatic repulsion would induce dissociation. This hypothesis is, however, incompatible with the finding that further peptide addition after dissociation causes reaggregation.

A documented ability of penetratin to translocate across lipid bilayers [10] can, nevertheless, explain the data presented here. Since aggregation apparently requires a certain penetratin density at the membrane surface, dissociation could be the result of peptide depletion from the outer leaflet of the lipid bilayer as a consequence of translocation to the interior of the liposomes. The lengthy aggregation/dissociation process might reflect either a slow peptide translocation or a slow equilibration process in response to rapid translocation of peptide molecules. All results presented in this work agree well with slow peptide translocation. When further penetratin addition is made after dissociation, the same aggregation/dissociation process is repeated as the newly added peptide equilibrates over the membrane. Also, translocation is consistent with the fact that dissociation no longer occurs if the P:L ratio is higher than $\sim$1:7 (data not shown). In this case, even if half of the peptide molecules initially associated with the liposomes translocate across the membrane, the peptide concentration on the outer leaflet is still high enough to keep the liposomes aggregated. The fact that addition of a second set of liposomes to the aggregates results in a rapid disaggregation does not contradict the slow peptide translocation hypothesis, since it merely reflects the rapid redistribution of penetratin from the outer leaflet of the aggregated liposomes to those recently added. It is, nevertheless, possible that the translocation of peptide molecules is fast. Penetratin internalization would lead to the build-up of a difference in potential between the lumen of the liposome and the exterior. This has to be

balanced by transport of ions across the membrane, which might be the rate-limiting step.

Quantitative evaluation of CD spectra of short peptides is complicated by several uncertainties related to e.g. their inherent flexibility, the chain length dependence and the influence of aromatic residues [24–26]. Therefore, the absolute numbers obtained in a secondary structure evaluation should serve merely as a tool for comparison of spectra and not be overinterpreted. In the studies of penetratin conformation when bound to phosphatidylglycerol vesicles, an α-helix to antiparallel β-sheet transition when going from low P:L ratios to high P:L ratios was observed. A similar behavior has been reported for the Alzheimer β-amyloid peptide(1–40) [27], apocytochrome *c* [28] and a synthetic peptide (HIV_{Arg}) representing the gp 41 N-terminus of LAV_{1a} [29]. In all these cases, as in ours, β-sheet formation is observed under conditions where aggregation of the liposomes occurs [28–30]. Thus, there seems to be a relationship between β-sheet structures and vesicle aggregation. To our knowledge, however, it has in no case been established whether aggregation of vesicles induces the formation of β-sheets or vice versa. In our system, this is difficult to deduce since these processes are simultaneous, as is evident from Fig. 2A. In the study of Magzoub et al. [11], antiparallel β-sheet structure was observed for penetratin bound to 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) liposomes. Based on our results, it is reasonable to assume that under the experimental conditions used in [11] (P:L ratio 1:10, DMPG), vesicle aggregation occurred. On the other hand, in the experimental setup (P:L ratio 1:325, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine:1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol molar ratio 70:30) employed by Drin et al. [12], no aggregation would be expected. In agreement with our results, they found the peptide to be in an α-helical conformation.

Although the change in CD spectrum upon increasing the P:L ratio is consistent with a conformational change between α-helix and antiparallel β-sheet structures, and in agreement with earlier reports on peptide CD changes in the presence of negatively charged liposomes [27,29], it is motivated to seriously consider error sources due to scattering or other optical artifacts. Measurements on particulate samples such as liposomes are complicated by a phenomenon known as 'absorption statistics', the origin of which is a significant probability with increasing size of particles that light may bypass between the particles in the light path through the sample. This leads to an attenuated apparent absorption, compared to the corresponding homogeneous sample, in regions of high absorbance. The artifact is also called Duysens flattening [31] because of the characteristic shape change it causes to an absorption band. Also in circular and linear dichroism the artifact causes decreased amplitudes in high-absorption regions, as observed for example with liposome samples [32].

The absorption spectra of liposomes in the presence of penetratin at a high P:L ratio (4.5 μM peptide to 25 μM lipid) show indications of both light scattering (turbidity) and absorption statistics (data not shown). The optical density increased typically from 0.20 to 0.25 at 250 nm (scattering) and decreased from 1.0 to 0.7 at 200 nm (absorption statistics). Hence, the decrease in CD amplitude observed at 200 nm and shorter wavelengths can have a considerable contribution from absorption statistics. However, there are strong arguments against that the CD spectral change could be entirely an artifact. Firstly, the change in amplitude around 210 nm, from strongly negative to almost zero CD, cannot be due to absorption statistics since the absorbance is here only moderate. The same holds true for changes at further longer wavelengths. Secondly, the control experiment in which a peptide sample was preceded by a scattering sample showed no change in the CD spectrum. Thus, spectral perturbation as a result of depolarization of the incident circularly polarized light caused by light scattering can be ruled out. Thirdly, penetratin has been shown to adopt an antiparallel β-sheet conformation when associated to a mixed 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine monolayer in a polarization modulation infrared reflection absorption spectroscopy study by Bellet-Amalric et al. [9]. Fourthly, in the study of HIV_{Arg} conformation by Rafalski et al. [29], the β-sheet conformation in the aggregated liposome samples, indicated by CD spectra very similar to those reported here, was also observed with infrared spectroscopy in peptide/lipid multilayers. In conclusion, although the influence of absorption statistics cannot be ruled out, the gross changes in shape and absolute amplitudes of the CD spectra of penetratin are in agreement with and indicate a change from an α-helical to an antiparallel β-sheet conformation at higher penetratin concentration and liposome aggregate formation.

Our findings shine new light on the membrane interactions of penetratin and may in the future contribute to an understanding of its translocation mechanism. Although the P:L ratios necessary for β-sheet formation may seem unrealistic for in vivo applications, there is a possibility that in heterogeneous biological membranes, locally high peptide concentrations are obtained transiently e.g. in regions with a high density of negatively charged lipids. Here, the existence of β-sheets of penetratin is conceivable.

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