Journal of Liposome Research, 15:29–47, 2005

ISSN: 0898-2104 print / 1532-2394 online
DOI: 10.1081/LPR-200064956

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Effect of Tryptophan Oligopeptides on the Size Distribution of POPC Liposomes: A Dynamic Light Scattering and Turbidimetric Study

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A chemical regulation of POPC liposome size distribution was investigated, based on the affinity of indole-containing compounds for phosphocholine membranes. In particular, tryptophan oligopeptides have shown interesting properties of size regulation, both when liposomes were formed in their presence and when the peptides were added to a preformed liposome suspension. Combining dynamic light scattering (DLS) and turbidimetric data, it was possible to show how such peptides had an influence on the size distribution of spontaneously formed liposomes prepared by the thin film hydration, reverse-phase evaporation and ethanol (or methanol) injection methods. In the presence of Trp-Trp or Trp-Trp-Trp, a disappearance of large vesicle aggregates was observed, as suggested also by light microscopy analysis. On the contrary, no effect was detected using extruded vesicles. Turbidimetric titration allowed the determination of the relative efficacy of the size regulators, Trp-Trp-Trp being about 20 times more powerful than the dimer, while the monomer had no effect. In addition, other indole-containing compounds and the antimicrobial peptide indolicidin were tested, showing similar behaviours. Discussing the results according to the current knowledge about the preference of Trp residues for interfacial regions in lecithin bilayers, this study confirms the relevant role of tryptophan in the biomembrane binding properties of many peptides and introduces a new behavior in the field of liposomes-peptides interactions.

Keywords dynamic light scattering, indolicidin, liposomes, size regulators, tryptophan, turbidimetry

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Introduction

The properties and the applications of liposomes are affected by their morphology and particle size distribution (PSD), which depend on the molecular and physico-chemical parameters as well as on the method of preparation. Up to the date, however, the size control of spontaneously formed liposomes remains still an empirical approach (in this context, ''spontaneously'' simply indicates ''without additional treatment''). For example, liposomes prepared by the lipid thin film hydration and by the reverse phase evaporation methods have a broad size distribution and are also morphologically heterogeneous (New, 1990). The regulation of the liposome size distribution can be accomplished mechanically, by extrusion through a polycarbonate membrane with a specific pore diameter, or—less efficiently—through sonication of the liposome suspension. Such procedures give the desired (altered) size distribution, because liposomes are kinetically trapped structure, and the ''relaxation'' toward a hypothetical equilibrium size distribution can be extremely slow.

On the other hand, as we have shown in previous papers (Domazou and Luisi, 2002; Stano et al., 2004), using the ethanol injection method it is possible to obtain a relatively narrow distribution of small unilamellar vesicles (SUV), simply modulating the experimental conditions. It would be very interesting, from fundamental and applicative point of views, to investigate the effect of *size distribution regulators*, i.e., molecules capable of modifying at some extent the liposomes size distribution.

Different chemicals can affect the liposome PSD, interacting at various degrees with the membrane. Studies are conducted on the liposome fusion/aggregation triggered by metal ions and fusogenic peptides. In the same way, the presence of drugs, polymers, sugars, and oligo- and polynucleotides can have an effect on the size distribution, shape, and morphology of vesicles. Studies with peptides and polypeptides, however, are of particular relevance, because of the importance of the interaction between lipids and proteins. At the best of our knowledge, also in this case there are no systematic investigations aimed at intentionally modulating the size of vesicles by chemicals addition.

Tryptophan residues play a crucial role on the biomembrane binding and functionality of peptides and proteins. Trying to elucidate the way that such compounds act, a large amount of work has been done on the interaction between various peptides/proteins and liposomes. Thus, the nature of the binding, the position, the location of tryptophan residues, as well as the binding effect on bilayer properties, such as stability, leakage, hole formation have been extensively studied. Furthermore, in nearly all membrane protein crystal structure known, Trp residues are not uniformly distributed, but they are generally located in a region that corresponds to the interfacial zone of lipid bilayer, suggesting their special function in the lipid-protein interaction.

More interestingly, some tryptophan-rich peptides, as tritrpticin (Lawyer et al., 1996; Schibli et al., 1999), indolicidin (Ladokhin et al., 1997; Schluesener et al., 1993; Selsted et al., 1992) and lactoferricin B (Bellamy et al., 1992) have an antimicrobial activity, and some authors have already recognized and studied this occurrence (Schibli et al., 2002). In particular, their mode of action has been discussed in terms of disruption of bacteria membranes, and several structural studies gave interesting information about the molecular details (Rozek et al., 2000; Schibli et al., 1999a,b, 2002).

In this report, we show the effect of simple Trp oligopeptides on the POPC liposomes size distribution, which can be conveniently studied by dynamic light scattering (DLS). In fact, the intensity-weighted size distribution (DLS raw data) is a sensitive probe for monitoring even small change in the high values side of the size

spectrum, due to the excellent sensitivity of DLS technique in the detection of large particles. In addition, and for comparison purpose, we studied some other hydrophobic oligopeptides, as Tyr-Tyr-Tyr and Phe-Phe, as well as some tryptophan containing peptides and other indole-containing compounds.

Our work suggests the existence of a *specific* interaction between phophatidylcholine and tryptophan residues, which probably modify the packing properties of the bilayer, with dramatic consequences on the intra- and intervesicles forces and interactions.

Materials and Methods

Chemicals

POPC (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was purchased from CHEMI S.p.A, Patrica (FR), Italy. The peptides Trp, Trp-Trp, Trp-Trp-Trp, Phe-Phe, Tyr-Tyr-Tyr, Glu-Trp, His-Phe-Arg-Trp · 2 AcOH and *N*-acetyltryptophanamide (NATA) were all from Bachem (Budendorf, Switzerland); tryptophan octyl ester hydrochloride (TOE), tryptamine and 5-methoxytryptamine were from Sigma (Buchs, Switzerland). Indolicidin (Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2) was from American Peptide Company. POPC and all the peptides were used without further purification. Boric acid, HEPES (*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), bicine (*N*,*N*-Bis(2-hydroxyethyl)glycine), sodium phosphates and NaCl were from Fluka; trifluoroacetic acid (Fluka, 98%) was distilled at atmospheric pressure. Ethanol absolute, methylene chloride, and diethyl ether were from J.T. Baker (Deventer, Holland). Aqueous solutions were always prepared with deionized and freshly purified water (Millipore Milli-Q apparatus).

Liposome Preparation

Thin Film Hydration Method (Comprehensive of Freeze-and-Thaw and Extrusion). POPC was dissolved in 20 mL of methylene chloride in a 100 mL round-bottom flask, the solvent was evaporated under reduced pressure (ca. 350 mbar) by means of a Rotavapor (Büchi, Switzerland), the resultant lipid thin film was then dried overnight under high vacuum. After hydration with the buffer solution (eventually containing the peptide) the milky suspension was mechanically stirred (15 min at about 120 rpm on the Rotavapor arm). When needed, 10 freeze-and-thaw cycles in liquid nitrogen were applied. Typically, 20 mL of 0.5 mM POPC liposome aqueous suspension were prepared.

To obtain extruded POPC liposomes, the milky suspensions derived from the freeze-and-thaw cycles were extruded (10 times for each pore size) by an Avestin Extruder® (Ottawa, Canada), using two stacked Whatman Nuclepore® polycarbonate membranes (Dietikon, Switzerland) with a definite pore size (400, 200, 100, or 50 nm). The final POPC concentration was estimated by the Ames' phosphate test (Ames, 1966).

Ethanol (or Methanol) Injection Method. POPC was dissolved in ethanol (or methanol) and an aliquot of the solution was fast injected, under vigorous magnetic stirring and by means of a Hamilton microsyringe, into an appropriate volume of aqueous solution (eventually containing the peptide). Typical conditions were 50 μL of 50 or 100 mM POPC ethanol solution into 4.95 mL of buffer (1:100 ''injection ratio'').

Reverse Phase Evaporation Method. A diethyl ether POPC solution was put in a 20 mL thick-wall cylindrical tube, then the appropriate amount of aqueous solution (eventually

containing the peptide) was fast injected, by means of a Hamilton microsyringe, into the organic lipidic solution. The resulting emulsion was sonicated (3 min) in a water-bath sonicator (Bandelin Sonorex RK100H, 140 W, 35 kHz), then the organic solvents evaporated under reduced pressure (ca. 400 mbar) using a Rotavapor (Büchi, Switzerland) until a gel was formed. Liposomes were obtained by vigorous mechanical stirring of the gel by a Vortex (Scientific Industries, Bohemia, NY, USA). Typical conditions were 1 mL of peptide containing buffer injected into 9 mL of 0.02 mM POPC organic solution, so that at the end of the evaporation, approx. 1 mL of liposome suspension with the desired peptide-to-lipid molar ratio was obtained.

When needed, liposome suspensions were centrifuged using an Eppendorf Micro Centrifuge, model 5415D, operating at 6000 rpm for 10 min (approx. 2400 *g*).

Unless specified otherwise, all experiments were carried out at room temperature.

Equilibrium Turbidity Measurement

A Cary 1E UV-Vis spectrophotometer, equipped with a software-controlled thermostat (all measurements at 25.0°C) and magnetic stirring facility was used. POPC liposomes for turbidity measurements were prepared by the ethanol injection method, as described before. The turbid solution was then diluted into the 1 cm quarz cell so that [POPC] = 0.05 mM. The sample was slightly turbid and tiny particulate was visible with a magnification lens. Then, aliquots of 10 μL of peptide solution were added, up to 100 μL or more, depending on the peptide concentration.

After each addition, the optical density (OD) at 500 nm was recorded—under stirring—until a stable signal was observed (readings were taken after 10–15 min). The equilibrium optical densities were then corrected for the dilution and plotted vs. the drug-to-lipid molar ratio. For each peptide three or two independent experiments were performed to evaluate the reproducibility, which can be estimated by the vertical bars (± standard deviation) shown in the reported plots. DLS analysis was performed before and after the addition. Data are presented as normalized relative turbidity, namely:

$$\text{relative turbidity} = \frac{A - A_\infty}{A_0 - A_\infty} \quad (1)$$

Where A, A_0, and A_∞ represent the optical density at a certain peptide-to-lipid ratio, at the beginning (no peptide) and at the end of the titration, respectively.

Time-Resolved Turbidity Measurement

POPC liposomes for turbidity measurements were prepared by the ethanol injection method and then diluted, as described before. A peptide buffer solution of 50 μL was fast added (the first 10 sec were lost to addition and shaking) and the optical density at 500 nm was followed for 60 min, under gentle magnetic stirring. The temperature was kept constant at 25.0°C.

Particle Sizing

Dynamic Light Scattering (DLS). The measurements were carried out on 1) an ALV home-assembled light scattering spectrometer made of a 25 mW He-Ne laser (Model 127, Spectra-Physics Lasers, Mountain View, Canada), an ALV DLS/SLS-5000 Compact Goniometer System (ALV, Langen, Germany), two SPCM-AQR avalanche photodiodes

(PerkinElmer Optoelectronics, Vaudreuil, Canada) and an ALV-5000 Multiple-tau Digital Correlator (ALV, Langen, Germany); 2) a Zetasizer 5000 (Malvern Instruments, Malvern, U.K.) consisting of a photomultiplier tube, a Malvern 7132 multibit digital correlator, and a 5 mW He-Ne laser. The cylindrical scattering cells were immersed in a fuzzy-thermostated decaline bath (ALV instrument) or in a Joule-Peltier controlled water bath (Zetasizer 5000), in both cases kept at 25.0°C. All the experiments were performed at the scattering angles 60, 90, and 120°; other settings were solvent viscosity 0.899 mPa · s, solvent refractive index 1.33. Due to the different refractive index and viscosity of the medium used (buffer or buffer/ethanol) compared to pure water, the size values calculated by fitting procedures must be intended as an approximation of the ''true'' size. Results are shown in terms of apparent hydrodynamic radius of vesicles, i.e., no correction was done to take into account the effect of concentration. Great care was taken to avoid the presence of dust in every step of the liposomal preparations, which were analyzed—unless specified otherwise—without any pretreatment.

The results obtained from the two instruments were comparable, especially for monomodal distributions. In the cases of intensity-weighted multimodal distributions, slightly different results were produced by the two instruments, mainly due to different signal sampling method, averaging procedures and inversion algorithm parameters.

In a dynamic light scattering experiment, the intensity (counts/second) and the autocorrelation function of the scattered light are measured. The particle size distribution (PSD), computed by an inverse Laplace transformation of the data, can be obtained with different algorithms. We used both the ILT and CONTIN procedures on the ALV-5000 instrument, as well as the NNLS (Non-Negative Least Squares, smoothing 0.005) and CONTIN procedures on the Malvern Zetasizer 5000. In the case of very broad size distribution all these methods are affected by some limitations; in the present work, however, emphasis was placed in the variations induced by the presence of size regulators. It is worthwhile to note, however, that all the conclusions shown are not dependent from the algorithm and instrument used, as well as from the eventual dilution applied to the sample to reduce or eliminate the multiple scattering.

Light Microscopy. Light micrographs were recorded with an Axioplan Zeiss Microscope (Germany), equipped with 40 × and 100 × objectives (in the latter case oil was put between the specimen and the lens), a CCD video camera module and connected to a computer. Images were acquired using DIC (Differential Interference Contrast) filter, memorized by the software Image Access (IMAGIC) version 1.55 for Windows 3.11, and digital processed (contrast/brightness/tonal range) using Adobe Photoshop 6.0 for Windows, for a better representation.

Peptide Analysis

In order to check the chemical stability of tryptophan oligopeptides in borate buffer, HPLC analyses of the peptides were conducted by HP 1050 Modules, HP 1050 Diode-Array Detector, and HP ChemStation software, equipped with a RP Nucleosil 100-5 C18 5 μm (25 mm, 4.6 mm i.d.) column. The mobile phase was composed by TFA 0.1% v/v in water (A) and TFA 0.1% v/v in acetonitrile (B); the elution was conducted using a constant flux of 0.8 mL/min according to the following program: 0 min, 100% A; 3 min, 100% A; 40 min, 20% A and 80% B; 45 min, 100% A. Under these conditions (injection volume of 5 μL, UV detection at 280 nm) Trp, Trp-Trp and Trp-Trp-Trp shown a retention time of 19.5, 25.5, and 29.5 min, respectively. In the case of Trp-Trp and Trp-Trp-Trp, together with the principal peak, one and two small peaks (1 and 1.5% on UV

basis) were present, revealing some impurity in the starting materials. The peptides were stable within 5 days; long-time stability was not checked.

Results

The effect of the size regulator (the peptide) on the liposome size distribution was investigated by DLS and indirectly by spectroturbidimetry, using two types of experimental setup. In the first series, liposomes were prepared in the presence of the peptide; in the second, the peptide was added to a preformed liposome suspension. What we observed as ''effect,'' a change in the DLS size distribution toward smaller particles, is supported, in the most evident cases, by the visual observation of a strong reduction of the turbidity, and of the absence of liposome aggregates sedimentation.

Liposomes Prepared in the Presence of the Size Regulator

Injection Method. Liposomes can be formed easily and conveniently by means of the ethanol injection method (Batzri and Korn, 1973), but also methanol (Domazou and Luisi, 2002) and other water-soluble solvent can be used. The method consists in the fast injection of a small aliquot of a lipid alcoholic solution into an aqueous phase. Depending on the lipid (alcoholic) concentration and on the injection ratio, vesicles with different size distribution are formed (Domazou and Luisi, 2002; Stano et al., 2004). In particular, at low lipid (alcoholic) concentration, a narrow population of small unilamellar vesicles arises. Increasing the lipid concentration in the stock ethanol solution, the liposome mean size and polydispersity increase, with tendency to form multimodal populations.

In Fig. 1 the size distributions of spontaneously formed POPC liposomes obtained by the ethanol injection method are shown (1:100 injection of a 100 mM POPC ethanol

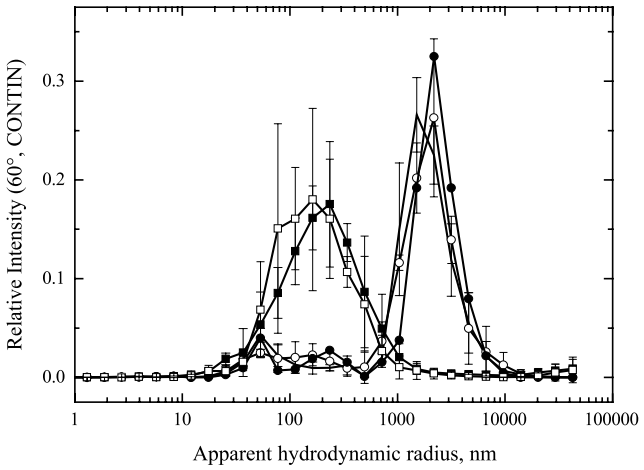

Figure 1. Size distributions of POPC liposome dispersions (0.1 M sodium borate, pH 8.5) prepared in the presence of different peptides by the ethanol injection method. A 100 mM POPC ethanol solution was injected (1:100) into the buffer solution containing the peptides, with vigorous stirring (total volume 5 mL). The bars represent the SD of three independent experiments. Control (——), 1 mM Trp (—○—); 10 mM Trp (—●—); 0.5 mM Trp-Trp (—■—); 0.33 mM Trp-Trp-Trp (—□—).

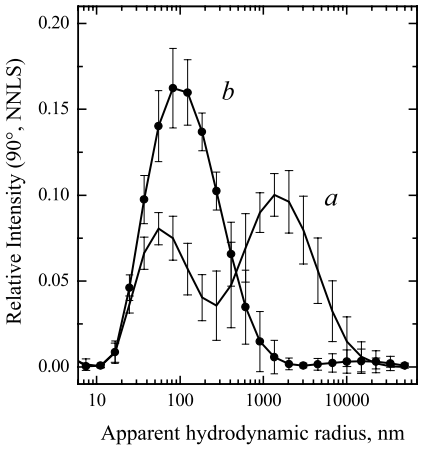

Figure 2. Size distributions of 0.5 mM POPC liposome dispersions (0.1 M sodium borate, pH 8.5) prepared in the presence of Trp-Trp 0.25 mM by the methanol injection method (final methanol 1% v/v) (*a*) control (——); (*b*) Trp-Trp effect (—●—). The curves shown (bars representing the standard deviation) are the averages of three independent experiments.

solution into 0.1 M sodium borate buffer, pH 8.5), as obtained by DLS. The curves show the existence of quite big particles with size between 1 and 10 μm. In the presence of Trp-Trp or Trp-Trp-Trp, at the same indole-to-lipid ratio, the particle size distribution is shifted toward smaller radius, between 100 nm and 1 μm. The effect was visible also with naked eyes, because after the injection a slightly opalescent dispersion come out. On the contrary, the monomer Trp did not have any effect, even used at high concentration. Likewise, if Phe-Phe and Tyr-Tyr-Tyr are used, no effect was observed (data not shown). When methanol was used instead of ethanol in the injection method, similar results (Fig. 2) were obtained. In addition, some hydrophobic indole-containing compounds, as tryptamine and 5-methoxytryptamine had the same effect as Trp-Trp and Trp-Trp-Trp (Table 1).

Ethanol injection method was also used for checking the size regulator properties of the five Trp-residues containing indolicidin, which was active at very low peptide-to-lipide molar ratio, with the usual reduction of the 1–10 μm DLS peak and parallel increase of smaller particles.

Lipid Film Hydration Method. With this method multilamellar and multivesicular vesicles are generally formed, together with a certain amount of large oligo- and unilamellar vesicles. The PSD of these dispersions is very broad, reflecting morphological heterogeneity. In many cases, after the hydration step, other operations are needed to control the liposome size distribution. Freeze-and-thaw, sonication, and extrusion are generally performed, depending on the aim of the preparation.

We investigated the effect of the Trp-containing peptides on the POPC liposomes as they are formed after film hydration as well as after the post-hydration procedure of freezing and thawing.

In Fig. 3, the PSD shift when POPC is hydrated with a borate buffer containing Trp-Trp-Trp is shown, in comparison with buffer alone. It was possible to observe the effect of size reduction before and after the freeze-and-thaw procedure. Interestingly, in the case of Trp-Trp, the effect was absent or it was very small before the freeze-and-thaw step, at

Table 1
Summary of the effect of tryptophan-containing peptides and other indole-containing compounds on the size distribution of POPC liposomes in 0.1 M sodium borate, pH 8.5

	Trp	Trp-Trp	Trp-Trp-Trp	Others
Lipid film hydratation[a]	no	no[g]	yes	—
Lipid film hydratation and freeze-and-thaw[b]	no	yes[h]	yes	—
Lipid film hydratation and freeze-and-thaw and extrusion (400 nm)[c]	—	—	no	—
Reverse phase evaporation[d]	—	yes[i]	—	—
Methanol injection[e]	no	yes	yes	Tryptamine and Methoxytryptamine: yes Tyr-Tyr-Tyr and Phe-Phe: no
Ethanol injection[e]	no[f]	yes	yes	TOE (*), NATA (**): yes Phe-Phe, Tyr-Tyr-Tyr, Glu-Trp and His-Phe-Arg-Trp: no

Vesicles were formed in a size-regulator containing buffer. ''Yes'' indicates the effect of shifting the size distribution toward smaller sizes; ''no'' indicates no effect. In all cases the ''effect'' is checked against a control experiment (buffer only). All measurements were conducted in triplicate to check reproducibility of effects and controls, with homogenous results.

[a]Typically 0.1–0.2 mM POPC; various peptide concentrations.

[b]10 cycles.

[c]10 times through two stacked polycarbonate membranes.

[d]Typically 0.25–1 mM POPC from a diethyl ether/buffer micro-emulsion; various peptide concentrations.

[e]Typically 0.15–1 mM POPC; residual alcohol 1–3% (v/v); various peptide concentrations.

[f]No effect up to 10 Trp-to-POPC molar ratio.

[g]No effect in sodium borate, HEPES, and bicine buffers at the same pH and ionic strength (8.5 and 18 mM, respectively).

[h]No effect in pure water, 20 mM NaCl, 12 mM sodium phosfate buffer (pH 6.6, ionic strength 18 mM), 0.1 M sodium phosphate (pH 6.6, ionic strength 177 mM). Effect in sodium borate, bicine, and HEPES buffers at the same pH and ionic strength (8.5 and 18 mM, respectively).

[i]Effect was detected also in 35 mM bicine buffer (pH 8.5, ionic strength 18 mM).

(*) Tryptophan octyl ester hydrochloride.

(**) *N*-acetyltryptophanamide; a small effect was detected.

least in the range of concentration explored. After the freeze-and-thaw step, the effect was clear (data not shown).

Reverse Phase Evaporation Method. In the reverse phase evaporation method (Szoka and Papahadjopoulos, 1978), a small volume of an aqueous solution is injected in an organic lipid solution (generally diethyl ether) so that a water-in-oil (micro)emulsion is formed, which is then transformed into a gel evaporating the organic solvent. From the

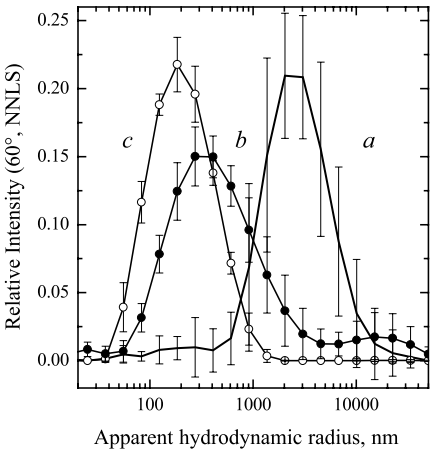

Figure 3. Size distributions of 0.5 mM POPC liposome dispersions (0.1 M sodium borate, pH 8.5) prepared in the presence of Trp-Trp-Trp 0.17 mM. (*a*) control (——); (*b*) effect before freezing-and-thawing (—●—); (*c*) effect after freezing-and-thawing (—○—). The curves shown (bars representing the standard deviation) are the averages of three independent experiments.

gel, vesicles are obtained by vortexing and eventually dilution. Applying this method, a broad population of large unilamellar and oligolamellar vesicle is generally obtained.

The presence of Trp-Trp in the buffer solution has an effect on the size distribution of the resultant POPC liposomes, see Fig. 4. In the control experiment a bimodal size distribution is obtained, which transforms in a rather wide monomodal population of smaller particles.

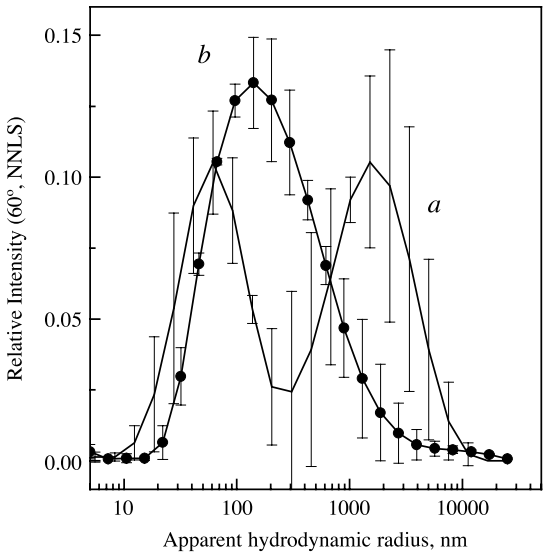

Figure 4. Size distributions of 0.26 mM POPC liposome dispersions prepared by the reverse phase evaporation method, in the presence of Trp-Trp (peptide-to-lipid ratio = 1:2). (*a*) Control (——); (*b*) Trp-Trp effect (—●—).

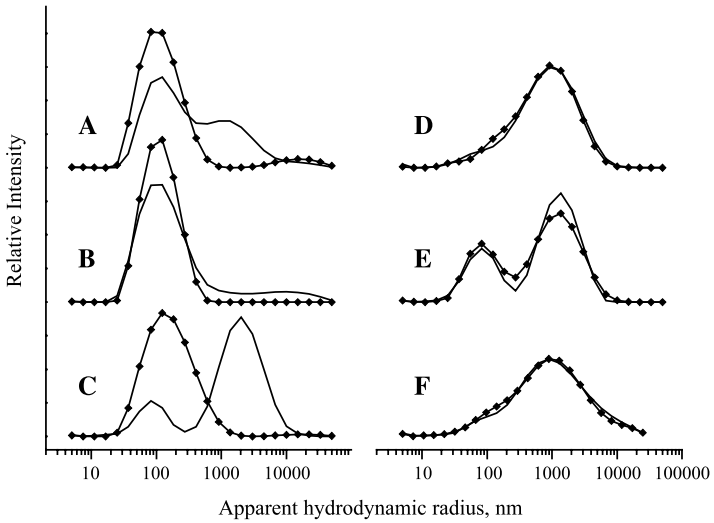

Figure 5. Size distributions of POPC liposome dispersions prepared by the thin film method, after freezing and thawing (10 cycles). Trp-Trp was present in the buffer from the beginning. In all cases: peptide-to-lipid ratio = 1:2. The curves shown are the average of three independent experiments; standard deviation not shown. (A) HEPES 21 mM, pH 8.5; (B) Bicine 35 mM, pH 8.5; (C) Sodium borate 100 mM, pH 8.5; (D) Sodium phosphate 12 mM, pH 6.6; (E) NaCl 18 mM; (F) water. Control (——); Effect (—◆—).

Other Buffers in the Case of Trp-Trp—Effect of Other Substances. In all experiments described, sodium borate has been used as buffer. When other buffers were used as bicine and HEPES buffers, keeping constant the pH, the counter-ion (sodium) and the ionic strength, Trp-Trp had an effect. On the other hand, when we use pure water or a saline solution or a sodium phosphate buffer (pH 6.6) we did not observe the effect on the size distribution of POPC liposomes as in the case of HEPES, borate, or bicine buffers measurements (Fig. 5).

Comprehensive results about the experiment in which the size regulator was present during the liposome formation are collected in Table 1. Note that we detected a strong size reduction effect also using tryptophan octyl ester (TOE), a membrane-soluble tryptophan derivative (Chattopadhayay et al., 1997; Ladokhin and Holloway, 1995). Weaker effect was detected using N-acetyl tryptophanamide (NATA), a non-ionizable tryptophan peptide-chain model compound. In contrast, no effect on the PSD was observed using other hydrophobic peptides, as Phe-Phe, Tyr-Tyr-Tyr; analogously, Glu-Trp and His-Phe-Arg-Trp did not change the size distribution.

Size Regulator Added to a Pre-formed Liposome Suspension

Spontaneously Formed Liposomes Having a Broad Size Distribution. Starting from a liposome suspension with a PSD that indicates the presence of big particles, we investigated the effect of addition of Trp-Trp and Trp-Trp-Trp on the preformed vesicles prepared with injection or film hydration methods. As Fig. 6 shows, both peptides have an effect on liposomes. When a liposome suspension prepared by the methanol injection method was treated with an equal volume of buffer solution, the initial bimodal size distribution (Fig. 6A) was not affected by 1:1 dilution. Vice versa, even small amount of

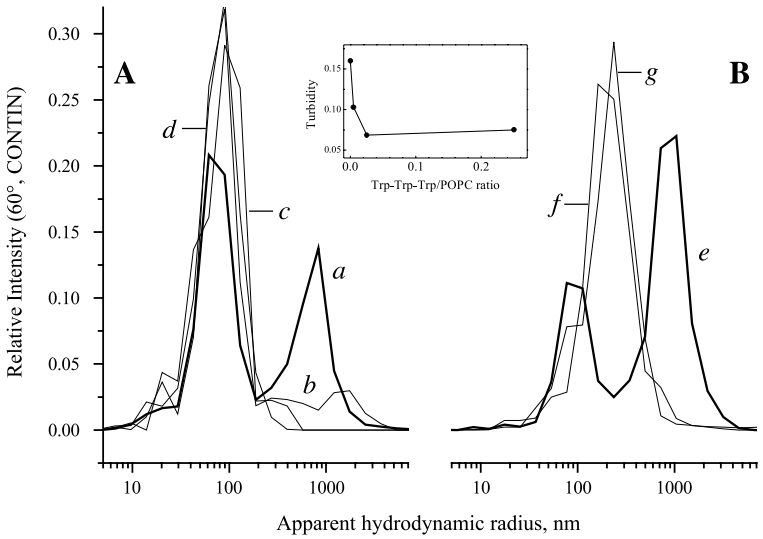

Figure 6. Size distribution of POPC liposome dispersions (0.1 M sodium borate, pH 8.5) after addition of Trp-Trp-Trp (left) or Trp-Trp (right) borate solution. (A) 0.5 mM POPC liposomes by methanol injection method (final MeOH 1.5% v/v): (*a*) borate addition (control); (*b*) 0.005 mM $(\text{L-Trp})_3$, peptide-to-lipid ratio 1:100; (*c*) 0.025 mM Trp-Trp-Trp, peptide-to-lipid ratio 1:20; (*d*) 0.25 mM Trp-Trp-Trp, peptide-to-lipid ratio 1:2. (B) 0.14 mM POPC liposomes by thin film method and bath sonication: (*e*) borate addition (control); (*f*) 0.068 mM Trp-Trp, peptide-to-lipid ratio 1:2; (*g*) 0.273 mM Trp-Trp, peptide-to-lipid ratio 2:1. Inset: optical density (turbidity) at 400 nm (path = 1 cm) of the Trp-Trp-Trp-containing samples.

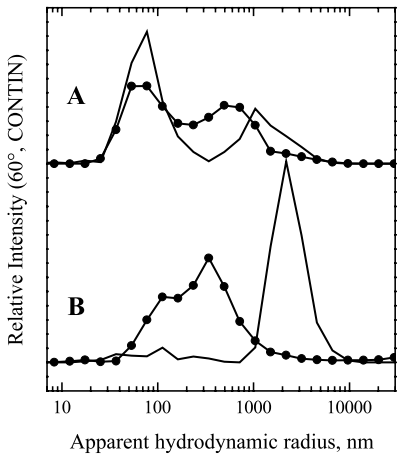

Figure 7. Size distributions of POPC liposome dispersions (0.1 M sodium borate, pH 8.5) before (——) and after (—●—) the addition of Trp-Trp 98 μM to the supernatant (A) and the sediment (B) fractions obtained by centrifugation (ca. 2400 *g*) of the initially produced dispersion (injecting 50 μL of a 100 mM POPC ethanol solution in 4.95 mL of buffer). DLS measurement conducted after 1:20 dilution.

Trp-Trp-Trp had a dramatic effect on the size distribution, reducing or destroying the peak relative to the big particles centered around 1 μm. In the same way, the turbidity at 400 nm was reduced (as well as the scattered light intensity), according to the size distribution changes (see inset), reaching a plateau after a drastic reduction as the peptide-to-lipid molar ratio was increased.

In the same conditions, tryptamine and 5-methoxytryptamine have the same effect of Trp-Trp-Trp, no effect was observed instead in the case of Tyr-Tyr-Tyr, a hydrophobic tripeptide (data not shown).

A similar behavior was observed in the case of Trp-Trp (Fig. 6B), using a liposome suspension prepared by the thin film hydration method. Also in this case, big particles disappear even at low peptide-to-lipid ratio.

In addition, the following experiment was performed. A liposome suspension prepared by the ethanol injection method (under conditions that big particles are produced, i.e., 100 mM POPC in ethanol, injected 1:100) was centrifuged to roughly separate very large particles from the smaller one. After separation, an aliquot of Trp-Trp was added to the supernatant and to the resuspended sediment fractions, and the consequent turbidity changes were monitored, as well as the initial (without peptide) and the final (with peptide) PSD. Interestingly, as shown in Fig. 7, the supernatant bimodal size distribution had a relatively small shift of the large size peak to lower values, without modification of the position of the small size peak. On the contrary, the sediment size distribution, consisting initially in one large size peak, was radically transformed to a new—shifted—size distribution, centered on smaller size values. The turbidity variations reflected these trends (data not shown).

Extruded and Small Unilamellar Liposomes. Vesicles of desired size are usually obtained by extrusion procedure (Hope et al., 1985), a mechanical reduction of size, the resultant vesicles being sometimes called VET (Vesicles by Extrusion Technique, for example VET_{100} indicates vesicles extruded by a ''100 nm-pore size'' polycarbonate membrane).

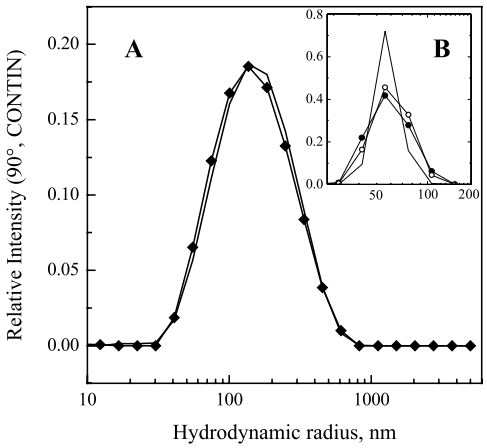

Figure 8. Size distributions of extruded POPC liposome dispersions (in 0.1 M sodium borate, pH 8.5), after the addition of the Trp-peptides. (A): 0.2 mM POPC ''400 nm'' after addition of buffer (——); Trp-Trp-Trp, peptide-to-lipid ratio = 1:2.8 (—◆—). Inset (B): 0.1 mM POPC ''100 nm'' after addition of: buffer (——); Trp-Trp, peptide-to-lipid ratio = 1:1 (—○—); Trp-Trp-Trp, peptide-to-lipid ratio = 1:3.3 (—●—).

When extruded liposomes were used as a substrate for the Trp-Trp-Trp or Trp-Trp action, no effect was detected, either using VET_{400} or VET_{100} samples (see Fig. 8). Likewise, if the conditions applied in the preparation of liposomes by the ethanol injection method led to a population of SUV, Trp-Trp had no effect on the size distribution (data not shown).

Turbidimetric Titration and Kinetics of the Effect. Spectroturbidimetry is a convenient method to monitor changes in the scattered light of colloidal suspension. Turbidity, scattered light, and size distribution are related by complex relationship (Khlebtsov et al., 2001; Matsuzaki et al., 2000; Pozharski et al., 2001; Shchyogolev, 1999), however, for diluted solutions of particles with small refractive index, it can be shown that turbidity τ (or the proportional optical density) is proportional to the scattered light (Hiemenz and Rajagopalan, 1997). We employed turbidimetry to investigate in a quantitative way the interaction of tryptophan oligopeptides with POPC liposomes; i.e., performing a turbidimetric titration by addition of aliquots of peptide solutions to a liposome suspension, see Fig. 9. When the buffer (control experiment; data not shown) or a monomer Trp solution was added, no change in the dilution-corrected turbidity was observed, even at high Trp-to-lipid ratio (curve *a*). On the contrary, if the dimer or the trimer solutions were used, the turbidity decreases, according to the amount of peptide added (curves *b* and *c*, respectively). In both cases the final turbidity value (as well as the final size distribution, data not shown) was approximately the same, but it was reached at different peptide-to-lipid molar ratio. In addition, whereas in the control and Trp experiments tiny particulate was still visible in the sample, in the case of Trp-Trp and Trp-Trp-Trp no particulate was present at the end of the titration. Curves *b* and *c* have an S-shaped behavior. We can characterize empirically the effect of the peptides by their

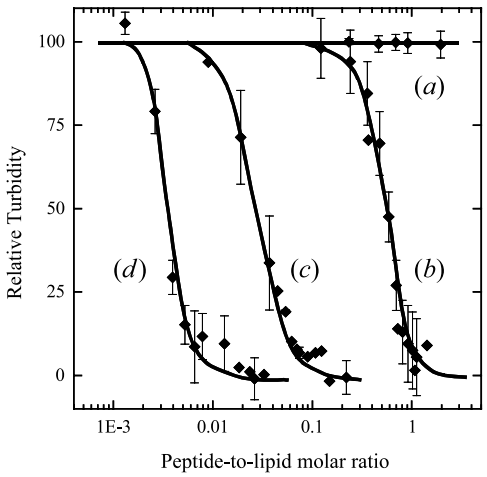

Figure 9. Turbidimetric titration of 50 μM POPC vesicles with Trp oligomers and indolicidin in 0.1 M sodium borate (pH 8.5); wavelength 500 nm. To a vesicle suspension (prepared by the ethanol injection method) small aliquots of peptides solutions were added. After each addition the equilibrium was reached within 10–15 min, as judged by the stability of the apparent optical density; reported data are corrected for the dilution and normalized; the error bars represent the S.D. of two or three experiments; the solid line was drawn to show the trend of the data points. (*a*) Trp; (*b*) Trp-Trp; (*c*) Trp-Trp-Trp; (*d*) indolicidin. Addition of buffer, as a control, did not produce a turbidity reduction (data not shown).

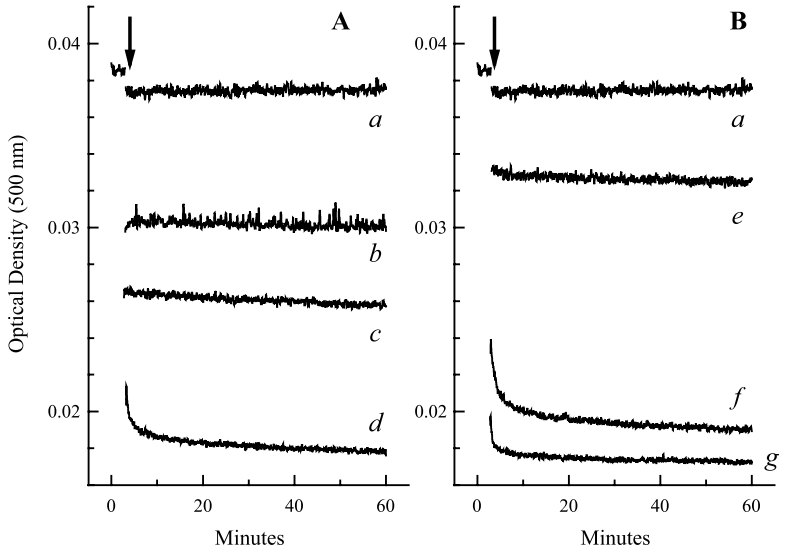

Figure 10. Time traces of the turbidity after addition of 50 μL of a Trp-Trp (A) and Trp-Trp-Trp (B) buffer solution to a 2.0 mL of 45 μM POPC liposome suspension, under stirring conditions. The arrows indicate the addition; overall mixing time ca. 10 s. Peptide-to-lipid ratio: 0 (*a*), 0.5 (*b*), 1 (*c*), 2 (*d*), 0.02 (*e*), 0.04 (*f*), 0.6 (*g*). The traces shown were smoothed using the built-in Origin 6.1 FFT filter (20 data points) for a better representation.

$R_{1/2}$* peptide-to-lipid molar ratio (or by the corresponding concentration); i.e., the peptide amount needed to half the total turbidity variation. The measured $R_{1/2}$* values are ca. 0.6 and 0.03 for Trp-Trp and Trp-Trp-Trp, respectively, showing that the trimer is about 20 times more active than the dimer, a value that cannot be explained only on the basis of the different *indole* content.

Indolicidin was also used in this test, producing a turbidity reduction, but the behavior was different when the sample was stirred, originating an upward drift after the initial turbidity decrease. As can be appreciated in Fig. 9 (curve *d*), indolicidin had $R_{1/2}$* ≈ 0.005. The process was also studied kinetically, following the turbidity changes with time after the peptide additions (Fig. 10). Both Trp-Trp and Trp-Trp-Trp cause a strong reduction of turbidity in the mixing time ($\sim$10 s), the process being very fast. The turbidity reduction is dependent on the amount of peptide added. The kinetics are characterized by a first very rapid phase present in all cases studied, in some cases mixed with a relatively slow phase (observed only at high drug-to-lipid ratios, see curves *d*, *f* and *g*, where the particles has to rearrange themselves in a greater manner), and by a third extremely slow phase that follow the initial fast changes. A quantitative study of this process should take into account the turbidity dependence from the number, shape, optical properties, *inter*-particle interactions and the size distribution of the particles present in the sample, a quite complex problem that will be not analyzed in this work.

Light Microscopy Analysis

The Effect of Trp-Trp and Trp-Trp-Trp on the Broad Size Distribution Was Also Studied by Light Microscopy. By injecting a concentrated (e.g., 100 mM) POPC ethanol or methanol solution into an aqueous phase, small unilamellar vesicles are formed as well as

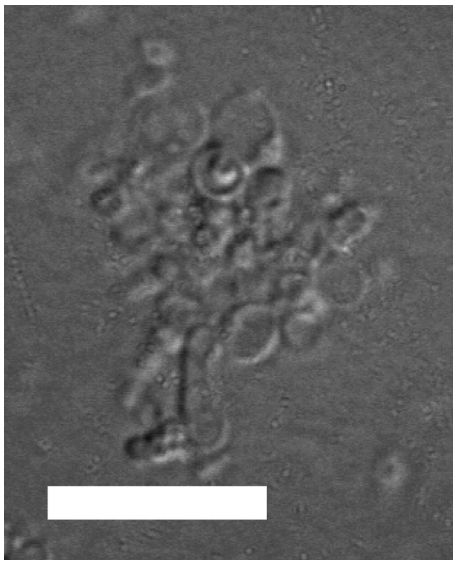

Figure 11. Light microscopy of a liposome aggregate, prepared by injection, under stirring, of an ethanolic 100 mM POPC solution into sodium borate buffer (0.1 M, pH 8.5). Final POPC concentration: 1 mM, ethanol 1% v/v. The picture has been taken with a 100 × objective, using DIC filter and software processed (contrast enhancement); the bar represents 10 μm.

vesicle clusters and partially hydrated lipid structures (Domazou and Luisi, 2002), see Fig. 11. In the presence of Trp-Trp-Trp such structures are not detected.

Light micrographs confirm scattering results, showing that DLS can be used—cautiously however, because the scatters should not sediment during the experiment—to determine the approximate size of such particles; in fact, as shown in Fig. 1, the estimated DLS size for the aggregates is around 1–10 μm.

Discussion

The change in the DLS intensity-weighted size distribution consists in the destruction of large particles and the consequent formation of smaller particles. As the light microscopy study shows clearly, together with the turbidity results and visual inspection, the observed phenomenon is the disappearance of liposome aggregates, formed during the hydration step of phospholipids. These aggregates are very large, their size is of the order of several microns, and they can be even seen as tiny particulates in the turbid liposome suspension. As we shown in a previous paper (Domazou and Luisi, 2002), liposome clusters are formed, depending on the conditions, by the (methanol) injection method. Similar particles are generally obtained when the film method is used or by the reverse phase evaporation method under certain conditions. We can ascribe the formation of the clusters to the imperfect hydration of lipids, due to the *local* high lipid concentration in the hydration step of liposome formation. In fact, highly concentrated POPC ethanol solutions produce such structures when injected in an aqueous medium, rather independently from the injection ratio (Domazou and Luisi, 2002; Stano et al., 2004). When liposome aggregates are not present (for example in a population of unilamellar or oligolamellar vesicles) the peptide-induced effect has not been observed, as we have shown above (Fig. 8).

In addition, a simple separation step (by mild centrifugation) of the raw suspension produces two systems, which show different response to the peptide addition (Fig. 7), namely a weak effect in the supernatant (aggregates-poor) fraction and a strong size reduction in the sediment (aggregate-rich) fraction.

Active peptides and derivatives show their effect either if present in the aqueous phase from the beginning or if added later, when liposomes are already formed, independently from the method of preparation. However, when the conditions used for the liposome preparation do not produce these aggregates, no effect was detected (for example injecting a low-concentration ethanol lipid solution in an aqueous buffer).

The kinetic of disaggregation is very fast, and the process can hardly be followed by normal spectrophotometric technique. Only for large turbidity decreases it was possible to detect a tail of a relatively slow process, which ends in any case within a few seconds after the peptide addition (Fig. 10). Moreover, the ''equilibrium'' turbidimetric titrations show that the decrease of turbidity and thus the amount of aggregates destroyed depends on the amount of peptide added to the suspension. In other words the process is not catalytic. DLS shows that even a small amount of Trp-Trp-Trp was able to disaggregate big particles leading to a radical change in the size distribution (Fig. 6A); this is easily understood considering the intensity-weighted character of the size distribution, in which big particles dominate the scattered light intensity, and their disappearance has a dramatic effect on the PSD curve.

About the mechanism of the observed effect and the corresponding driving-force the following qualitative conclusions can be drawn from our results. We must consider that the effect is operative at two levels: 1) the molecular level, where a transient or stable interaction between the lipid and the peptide molecules must occur; 2) the ''colloidal'' level, where this interaction triggers an effect, i.e., intra- and interparticle behavior.

The interaction of Trp-based short oligopeptides with POPC membranes has been thoroughly characterized and this subject is well documented in the literature (Jacobs and White, 1986). However, at the best of our knowledge, there are no reports on their effect on the liposome size distribution. In addition, despite a large number of publications about triggering of vesicle aggregation and/or fusion, studies on the disaggregation of vesicle clusters are missing.

The oligopeptide effect on the PSD reflects a molecular interaction between the indole-containing compounds and the phosphatidylcholine mojety. For example, Wimley and White showed that Trp-residues of peptides, which interact with bio-membranes, are placed often in the ''broad, dynamic and chemically heterogeneous'' interfacial region (Yau et al., 1998). The ''aromatic'' nature of indole (comprehensive of dipole and quadrupole, H-bonding and cation-π interaction) leads to the preference of tryptophan for membrane interfaces, being squeezed out from water due the high hydrophobicity and not accomodated in the bilayer core due to the cohesive repulsion of the acyl chains. The interaction between phosphatidylcholine and Trp-containing oligopeptides probably led to a modification of packing in the membrane, triggering a morphological transformation that ends up with the disaggregation of liposomes clusters. It is known, in fact, that upon binding of peptides, bilayers change their physical properties (Brown and Huestis, 1993; Jacobs and White, 1989). Our finding that Trp per se does not interact with POPC liposomes, even at high concentration, is in agreement with previous data (Schmitz et al., 2000). On the contrary, Trp-Trp and especially Trp-Trp-Trp are active at relatively low concentration. This can be explained with the enhanced hydrophobicity of two or three connected tryptophan units compared to the single one, which should increases the adsorption on the membrane. A similar effect was

shown by the membrane soluble tryptophan octylester (TOE, see Table 1), where, however, the additional presence of the octyl chain does not permit a simple comparison of the data. Notice, however, that using NATA a size shift effect was observed (see Table 1), suggesting that removing the Trp charges, i.e., increasing hydrophobicity, produces a compound with size-regulating property, having higher affinity for the membrane. The PSD shifting effect is caused by the interaction of Trp-Trp and Trp-Trp-Trp with POPC liposomes, the trimer being effective at lower concentration due to the increased number of indole ring, and hydrophobicity. However the effect can be synergic, in the sense that Trp-Trp-Trp is not only more effective due to the higher partition on the bilayer respect to the dimer, even after indole normalization, but also because the property of ''size regulator'' might arise from the *connected* triple indole chain. Using indolicidin, which contains five indole rings, a minor amount of peptide is needed to develop the same effect, as Fig. 9 shows clearly. We conclude that higher Trp oligomers can have a stronger efficacy.

The buffer effect on the Trp-Trp action (see Table 1 and Fig. 5) needs deeper investigations and it is of no easy interpretation, because of the subtle interaction of ions with the phospholipid-water boundary, and their effect on energetic and mechanics of membranes; however it indicates a complex mechanism in which bilayer and ions hydration, peptide partition, and ionization contribute to the final effect. Another exceptional behavior is presented in Table 1 (first row): the absence of regulation of Trp-Trp after simple film hydration, while the trimer is well active in the same conditions. The difference disappears after the freeze-and-thaw procedure, which provides for solute partitioning (inside vesicles) and membrane reorganization. The reasons of this anomaly are not understood.

At similar peptide-to-lipid ratios, Phe-Phe and Tyr-Tyr-Tyr showed no effect on the size distribution. This can be explained, at least qualitatively, taking into account the different hydrophobicities of the Trp, Phe, and Tyr residues. According to Wimley and White (Wimley and White, 1996) the three aromatic residuals show the higher affinity for phosphatidylcholine membrane, when compared with the other amino acid residues; in addition the relative *interfacial* hydrophobicity is Tyr < Phe < Trp, Trp having a free energy change (bilayer-to-water) two times larger than the other two residues. Note that other hydrophobicity scales, as those of Kyte-Doolittle (Kyte and Doolittle, 1982) and Engelman-Steitz-Goldman (Engelman et al., 1986), place Trp and Tyr in the middle and Phe on the high-hydrophobicity side.

Finally, it is noteworthy that in all cases (Figs. 1–7) the final size distribution is a broad band centred at ca. 200 nm, rather independently from the method of preparation, suggesting that—in addition to the disaggregating effect—Trp-oligopeptides could have a ''real'' size regulation effect. In fact, a more intriguing mechanism can be operative, based on a complete rearrangement of the bilayer, driven by the peptide binding.

In this paper, using DLS and turbidimetry, we report a novel behavior in the field of peptide-lipid interactions. A more comprehensive study—using different techniques—is under progress in our laboratory, with the aim of deeper examining and better quantifying the effect. As addressed in the introduction, the chemical regulation of the liposome size distribution would represent a point of extreme interest in the liposome technology. Further, the additional presence of the peptide would also slightly complicate the preparation process, which would have, however, the advantage of mildness. The relevance of the phenomenon in the fundamental and applied research can be great (with its potential importance for understanding the action of tryptophan-rich antimicrobial peptides) and further investigations are needed.

Acknowledgments

We acknowledge Dr. Martin Müller (Institut für Angewandte Physik, ETH Zürich) for the use of the microscope, Dr. Richard M. Thomas (Institut für Polymere, ETH Zürich) for useful suggestions about the HPLC analysis of Trp oligopeptides.

References

Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. In: Neufeld, E. F., Ginsberg, V., eds. *Methods in Enzymology*. Vol. 8. New York: Academic Press, pp. 115–118.

Batzri, S., Korn, E. D. (1973). Single bilayer liposomes prepared without sonication. *Biochim Biophys. Acta.* 298:1015–1019.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta.* 1121:130–136.

Brown, J. W., Huestis, W. H. (1993). Structure and orientation of a bilayer-bound model tripeptide. A ^{1}H-NMR study. *J. Phys. Chem.* 97:2967–2973.

Chattopadhayay, A., Mukherjee, S., Rukmini, R., Rawat, S. S., Sudha, S. (1997). Ionization, partitioning and dynamics of tryptophan octyl ester: implications for membrane-bound tryptophan residues. *Biophys. J.* 73:839–849.

Domazou, A. S., Luisi, P. L. (2002). Size distribution of spontaneously formed liposomes by the alcohol injection method. *J. Liposome Res.* 12(3):205–220.

Engelman, D. M., Steitz, T. A., Goldman, A. (1986). Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15:321–353.

Hiemenz, P. C., Rajagopalan, R. (1997). *Principles of Colloid and Surface Chemistry*. 3rd ed. New York: Marcel Dekker, Inc., pp. 209–210.

Hope, M. J., Bally, M. B., Webb, G., Cullis, P. R. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55–65.

Jacobs, R. E., White, S. H. (1986). Mixtures of a series of homologous hydrophobic peptides with lipid bilayers: a simple model system for examining the protein-lipid interface. *Biochemistry* 25:2605–2611.

Jacobs, R. E., White, S. H. (1989). The nature of the hydrophobic binding of small peptides at the bilayer interfaces: implications for the insertion of transmembrane helices. *Biochemistry* 28:3421–3437.

Khlebtsov, N. G., Kovler, L. A., Zagirova, S. V., Khlebtsov, B. N., Bogatyrev, V. A. (2001). Spectroturbidimetry of liposome suspensions. *Colloid J.* 63(4):491–498.

Kyte, J., Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

Ladokhin, A. S., Holloway, P. W. (1995). Fluorescence of membrane-bound tryptophan octyl ester: a model for studying intrinsic fluorescence of protein-membrane interaction. *Biophys. J.* 69:506–517.

Ladokhin, A. S., Selsted, M. E., White, S. H. (1997). Bilayer interactions of indolicidin, a small antimicrobial peptide rich in tryptophan, proline, and basic amino acids. *Biophys. J.* 72:794–805.

Lawyer, C., Pai, S., Watabe, M., Borgia, P., Mashimo, T., Eagleton, L., Watabe, K. (1996). Antimicrobial activity of a 13 amino acid tryptophan-rich peptide derived from a putative porcine precursor protein of a novel family of antibacterial peptides. *FEBS Lett.* 390:95–98.

Matsuzaki, K., Murase, O., Sugishita, K., Yoneyama, S., Akada, K., Ueha, M., Nakamura, A., Kobayashi, S. (2000). Optical characterization of liposomes by right angle light scattering and turbidity measurement. *Biochim. Biophys. Acta.* 1467:219–226.

New R. R. C. (1990). *Liposomes: A Practical Approach*. The Practical Approach Series. New York: IRL Press at Oxford University Press.

Pozharski, E. V., McWilliams, L., MacDonald, R. C. (2001). Relationship between turbidity of lipid vesicle suspensions and particle size. *Anal. Biochem.* 291:158–162.

Rozek, A., Friedrich, C. L., Hancock, R. E. (2000). Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. *Biochemistry* 39:15765–15774.

Schibli, D. J., Hwang, P. M., Vogel, H. J. (1999a). Structure of the antimicrobial peptide tritrpticin bound to micelles: a distinct membrane-bound peptide fold. *Biochemistry* 38:16749–16755.

Schibli, D. J., Hwang, P. M., Vogel, H. J. (1999b). The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. *FEBS Lett.* 446:213–217.

Schibli, D. J., Epand, R. F., Vogel, H. J., Epand, R. M. (2002). Tryptophan-rich antimicrobial peptides: comparative properties and membrane interactions. *Biochem. Cell. Biol.* 80:667–677.

Schluesener, H. J., Radermacher, S., Melms, A., Jung, S. (1993). Leukocytic antimicrobial peptides kill autoimmune T-cells. *J. Neuroimmunol* 47:199–202.

Schmitz, A. A. P., Ulrich, A., Vergères, G. (2000). Membrane binding of MARCKS-related protein studied by tryptophan fluorescence spectroscopy. *Arch. Biochem. Biophys.* 380(2):380–386.

Shchyogolev, S. Y. (1999). Inverse problems of spectroturbidimetry of biological disperse systems: an overview. *J Biomed Opt* 4(4):490–503.

Selsted, M. E., Novotny, M. J., Morris, W. L., Tang, Y. Q., Smith, W., Cullor, J. S. (1992). Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J. Biol. Chem.* 267:4292–4295.

Stano, P., Bufali, S., Pisano, C., Bucci, F., Barbarino, M., Santaniello, M., Carminati, P., Luisi, P. L. (2004). Novel camptothecin analogue (Gimatecan)-containing liposomes prepared by the ethanol injection method. *J. Liposome Res.* 14(1–2):87–109.

Szoka, F., Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U. S. A.* 75:4194–4198.

Wimley, W. C., White, S. H. (1996). Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nat. Struct. Biol.* 3(10):842–848.

Yau, W. M., Wimley, W. C., Gawrisch, K., White, S. H. (1998). The preference of tryptophan for membrane interfaces. *Biochemistry* 37:14713–14718.