Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 60 (2003) 591–600
Talanta
www.elsevier.com/locate/talanta

Interaction of a laminin derived peptide with phosphatidyl choline/phosphatidyl glycerol

F. Reig [a,*], P. Sospedra [b], A. Juvé [a], L. Rodriguez [c]

[a] Department of Peptides, Institute for Biological and Environmental Chemistry, Jordi Girona 18, 08034 Barcelona, Spain
[b] Physicochemical Department, Faculty of Pharmacy, University of Barcelona, Avgda. Joan XXIII s/n, 08028 Barcelona, Spain
[c] Pharmacology Department, Faculty of Medicine, Casanova 143, 08018 Barcelona, Spain

Received 17 June 2002; received in revised form 17 February 2003; accepted 23 February 2003

Abstract

The physicochemical interactions between an active peptide sequence derived from laminin and phospholipids have been studied. The main aim is to determine the suitability of this peptide fragment to be linked to liposome's with the purpose to develop targeting devices. Results indicate that this peptide is able to insert in lipid monolayers and also to form monomolecular layers at an air/water interface. Besides, miscibility studies carried out through compression isotherms of mixed monolayers [dipalmitoyl phosphatidylcholine (DPPC)/peptide], indicate a strong interaction at 60–80% DPPC molar composition. Studies carried out with lipid bilayers indicate that the interaction is restricted to the external face of the vesicles. Moreover, the presence of this peptide in the incubation media promotes a low level of carboxyfluorescein (CF) leakage and no fusion of vesicles. These results indicate that the association of this sequence to vesicles do not produce damage of the bilayer and can be used as potential targeting vector.

Keywords: Laminin; Phosphatidyl choline; Phosphatidyl glycerol

1. Introduction

Adhesion processes are closely involved in cell proliferation and dissemination during tumor growing and metastases development [1,2]. Knowledge accumulated during the last years indicates that there are several proteins acting either as ligands or receptors involved in this process. Laminin and fibronectin are important structural elements of the human basement membrane whose receptors, integrins, are overexpressed in proliferant cells [3–5]. This suggests that active peptide sequences of these proteins could be useful both, to target drugs or to block receptors interfering in the biological events [6–8].

The most usual procedures to attach peptides or proteins to liposomal surfaces [9–11], involve the incubation of activated vesicles with the targeting vector in order to establish a chemical link between both entities. Nevertheless, it is well known, that some molecules after incubation with liposomes promote physicochemical changes

* Corresponding author. Tel.: +34-93-400-6100; fax: +34-93-204-5904.
E-mail address: fripfr@iiqab.csic.es (F. Reig).

0039-9140/03/$ - see front matter
doi:10.1016/S0039-9140(03)00186-3

affecting the ordered packing of bilayer lipid components. For this reason it is convenient, before starting the preparation of liposomes, to carry out a physicochemical study on the interactions between lipids and the selected targeting molecule.

Following this assumption, in this paper the synthesis and physicochemical study of a non-apeptide containing the active laminin sequence, SIKVAV, located in the E-8 region of this protein [5], is described.

Techniques used are based on monomolecular layers, spread on water/air interfaces, whose surface activity was determined in a Langmuir balance. Besides, interaction with bilayers was controlled through changes in different properties such as microviscosity, leakage of entrapped molecules and tendency towards membrane fusion. All the results indicate that the interaction of this sequence with lipids is soft and do not destabilize vesicle's structure. This peptide sequence can thus be used safely in the preparation of targeted liposomes.

2. Materials and methods

2.1. Chemicals

Fmoc aminoacids, resin and reagents were from Novabiochem. Lipids: dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG), chloroform, and fluorescent probes 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and 1-anilino-8-naphthalene sulfonic acid (ANS), were purchased from Sigma. Carboxyfluorescein (CF), from Eastman-Kodak was purified by column chromatography. *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dioleoyl phosphatidyl ethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-dioleoyl phosphatidylethanolamine (Rh-PE) were from Avanti.

2.2. Synthesis

Peptide was synthesized, manually, by the solid phase method on a Rink-MBHA resin type following the Fmoc/OBut strategy. Diisopropyl carbodiimide/*N*-hydroxibenzotriazol were used as coupling agents. Fmoc deprotection was carried out with DMF/piperidine (8:2). Cleavage from the resin and side-chain deprotection were achieved treating the resin with trifluoroacetic acid containing 5% water. Peptide was precipitated and washed with cold diethyl ether, dissolved in 10% acetic acid and lyophilized. Samples were purified by semipreparative HPLC, working with C_{18} columns and mixtures water/acetonitrile from 25 to 35% ACN. The identity of the product was confirmed by electrospray-mass spectrometry and amino acid analysis.

2.3. Surface pressure measurements

Surface activity and insertion in monolayers were measured in a Langmuir Balance (KSV 5000). A flat cylindrical Teflon cuvette of 70 ml capacity full of phosphate buffer saline was used for these experiments. During the measurements the temperature was kept at $22 \pm 1\ ^{\circ}C$, and the subphase was continuously stirred with a magnetic bar. Different volumes of a concentrated peptide solution (10^{-3} M) were injected through a lateral hole drilled on the cuvete wall and surface pressure increases recorded.

For insertion experiments, small drops of lipid/chloroform solutions (mg ml^{-1}), were spread on the water surface till reaching the desired initial surface pressure (5, 10 and 20 mN m^{-1}). Once the monolayer was stable (surface pressure remained constant), the peptide was injected as before. Details are given in [12].

2.4. Compression isotherms

Pressure–area isotherms were performed in the same device, equipped with a Wilhelmy platinum plate, using a rectangular Teflon trough (surface area 17 000 mm^2, volume 1000 ml). DPPC and peptide were dissolved in chloroform and DMSO, respectively, at 1 mM concentrations. Different volumes of both solutions were mixed in order to obtain the desired molar compositions. The compression isotherms, either pure DPPC, peptide, or mixed monolayers spread on phosphate buffer saline pH 7.4, were recorded, after allowing 15

min for stabilization. The monolayer was compressed (symmetrical compression), with an area reduction rate of 60 mm^2 min^{-1}. The films were compressed up to their collapse pressure. Each run was repeated three times and the reproducibility was ± 0.01 nm^2 per molecule. All experiments were performed at 294 ± 1 K [12].

2.5. Fluorescence experiments

Microviscosity of bilayers was determined through fluorescence polarization changes (Aminco Bowman spectrofluorimeter) of DPH and ANS probes. Liposomes composed of DPPC (20 mM) were prepared by the thin film method.

Briefly, a DPPC solution in chloroform was evaporated in a rotaevaporator and last traces of solvent were eliminated with an oil pump (2 h). Dry lipids were hydrated with an isotonic sodium acetate solution adjusted to pH 7.4. Suspensions were submitted to ultrasounds probe sonication in order to achieve diameters in the range of 100 nm. Aliquots of liposomes were incubated with the fluorescent probe (10 μM), for 30 min at 60 °C followed by incubation with peptide for 5 min at room temperature. DPPC/Probe molar relationship was in the range 250–350 per l. Polarization of DPH or ANS main band was measured in the temperature interval from 25 to 55 °C; details are given in [13].

Fusion of bilayers was determined measuring the resonance energy transfer (RET) induced on two liposomal populations labeled with either 0.6% *N*-NBD-PE or 0.6% *N*-Rh-PE. As reference, for 100% fusion, liposomes containing 0.3% of both fluorophores in the same vesicle were used. Vesicles of DPPC were prepared (as described before), including one of the fluorescent probes, and the resulting NBD and Rh-vesicles were mixed in a 1:1 mol ratio. Lipid mixing was determined monitoring the decrease in NBD-PE signal. The protocol followed is essentially the same described in [14].

Leakage of entrapped CF was determined as described in [13], from CF loaded liposomes. The increase of fluorescence intensity caused by its dilution upon leakage from a self-quenching concentration, was measured after different incubation times. The experiments were carried out at 21 °C. Results are expressed as the percent of the intensity obtained after Triton X-100 (10% in water) addition to completely disrupt the vesicles.

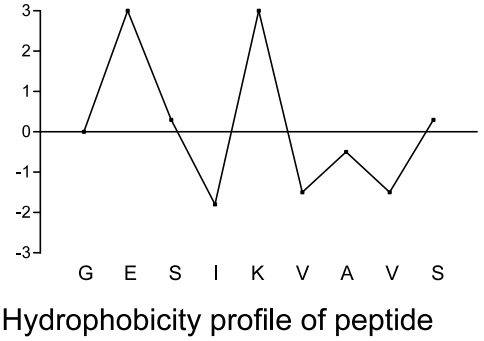
3. Results

3.1. Synthesis

The peptide sequence, $GESIKVAVS_{NH2}$, was designed based on the active fragment SIKVAV. Amino acid residues incorporated to the amino and carboxy terminal ends were introduced in order to improve water solubility. This is an important requirement for "in vitro" and "in vivo" experiments (see hydrophobicity profile Scheme 1). The synthesis was carried out following the solid phase methodology (Fmoc/OBut strategy) on a *p*-methyl benzhydrylamine resin in order to obtain the peptide as carboxamide form. Yield on crude peptide was 90%. Crude material was 85% pure according to peak area of HPLC chromatogram. Total yield of synthesis after preparative HPLC purification was 75% on pure peptide. The chromatogram of purified peptide is given in Fig. 1. Inset corresponds to its mass spectra.

3.2. Surface activity

Was determined for different subphase peptide concentrations, ranging between 6.3 and 50.7 μM, in order to find the saturation concentration (25.25



Scheme 1. Hydrophobicity profile of peptide $GESIKVAVS_{NH2}$.

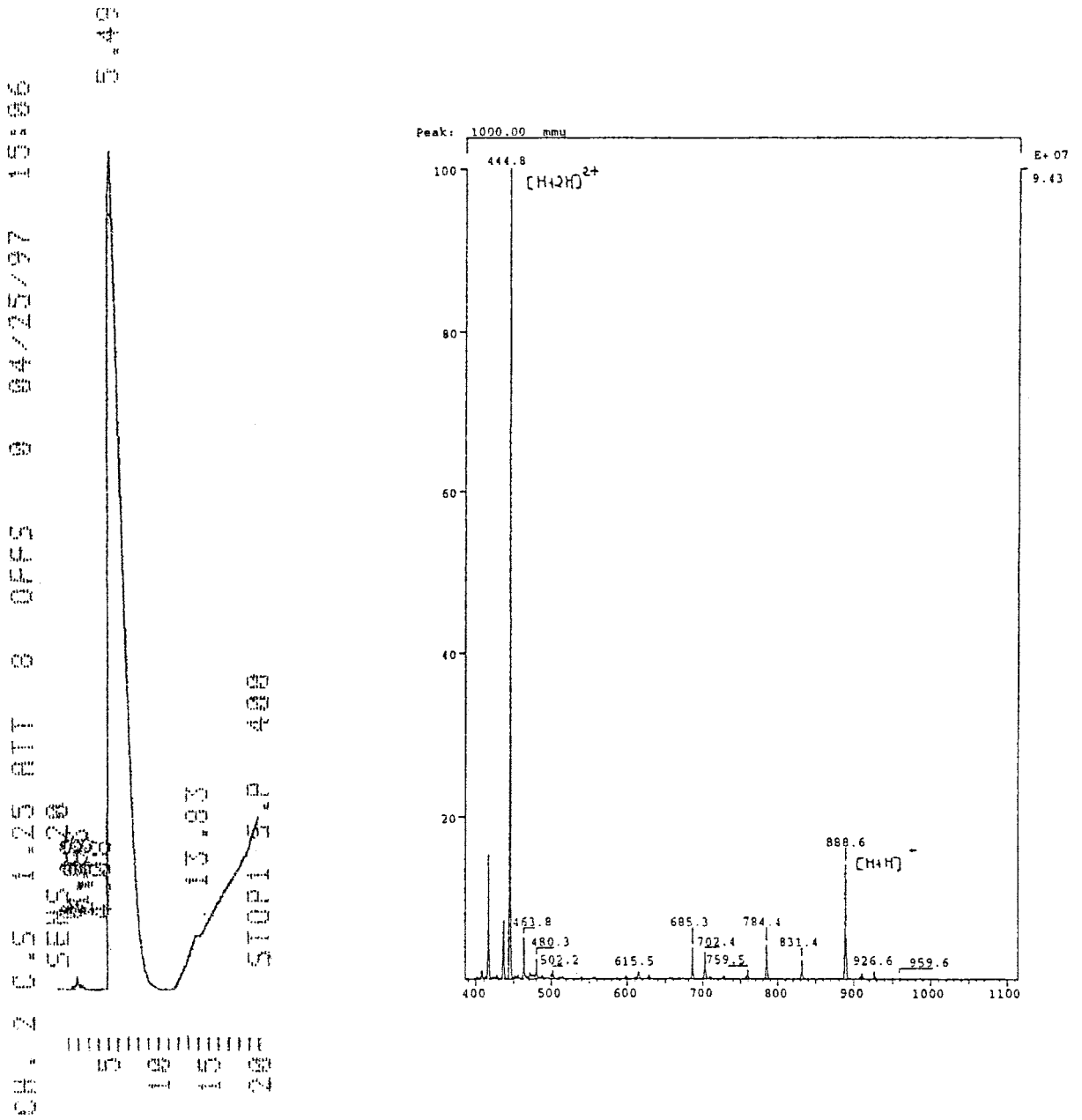


Fig. 1. HPLC chromatogram of purified GESIKVAVS; inset, mass spectra.

μM). As peptide sequence has an overall positive charge the influence of subphase pH on its surface activity was also determined. To this end experiments were also carried out with subphases adjusted to pH values 4.5 and 8.5. The time course of adsorption processes are given in Fig. 2. The kinetics were similar for subphases adjusted to acid or basic pH values, but on the contrary for neutral subphases, the surface pressure increased more slowly, although final values were in the same

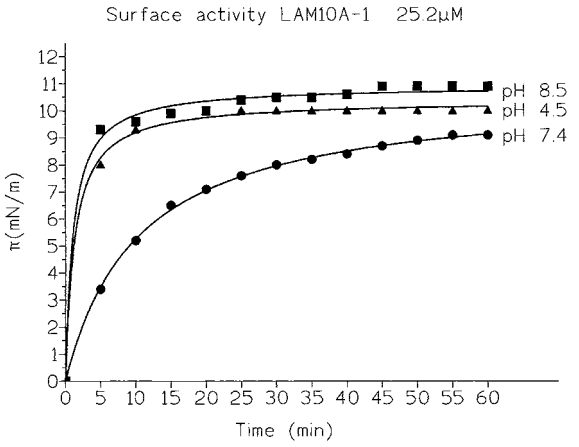


Fig. 2. Kinetics of adsorption of peptide molecules at the air/water interface. Peptide concentration in the sub-phase 25.2 μM. ■, pH 8.5; ●, pH 4.5; ▲, pH. 7.4.

range for all of them. A possible explanation for these differences can be that at extreme pH's some polar groups are non-ionized while at neutral pH the peptide is in zwiterionic form, rendering in this way a more hydrophilic molecule. Nevertheless, differences were very small (probably due to the overall high hydrophilicity of the peptide), and at the end of the process pressure increases were of the same order.

Surface excess (Γ) and area/molecule (A) at equilibrium were calculated applying (Eq. (1)) for the Gibbs adsorption isotherm and (Eq. (2)). Numerical values are given in Table 1.

$$\Gamma = 1/RT \times \Delta\Pi/\Delta \ln c \quad (1)$$

$$A = 1/\Gamma \times N_A \quad (2)$$

where R is the gas constant, (8.31 J K^{-1} mol^{-1}); T is the temperature, (294 K); c is the peptide concentration in the subphase; ΔΠ, is the pressure achieved after 60 min; N_A is the Avogadro's number.

Table 1
Surface excess and area/molecule (peptide concentration: 25.25 μM)

pH	4.5	7.4	8.5
Γ (mol m^{-2})	4.1×10^{-6}	3.7×10^{-6}	4.4×10^{-6}
A (nm^2 mol^{-1})	0.40	0.44	0.37

One can appreciate that the pH of the subphase has a limited influence in both parameters. Minimal Γ values correspond to neutral subphases and as a consequence the area occupied per molecule is slightly higher.

Moreover, these results are in agreement with the area molecule values calculated from the compression isotherms experiments as will be described in following paragraphs.

3.3. Monolayer experiments

The interaction of peptide with lipid monolayers was monitored by measurement of the surface pressure increase in a constant area set-up. This effect can be related to the degree and nature of penetration. The injection of GESIKVAVS, at the saturating concentration, under DPPC and DPPG monolayers gave rise to a surface pressure increase, which reached the stable level after 60 min Fig. 3 shows the dependence of the final pressure increase on the initial surface pressure of the monolayer. As a common trend pressure increases are lower for high initial pressures indicating a more restricted penetration. At all initial pressures insertion was slightly better for DPPC monolayers. Besides, the presence of peptide molecules in the subphase results in a lower pressure increases, in DPPG monolayers, because its molecules intercalate into the liquid-expanded DPPG and screen

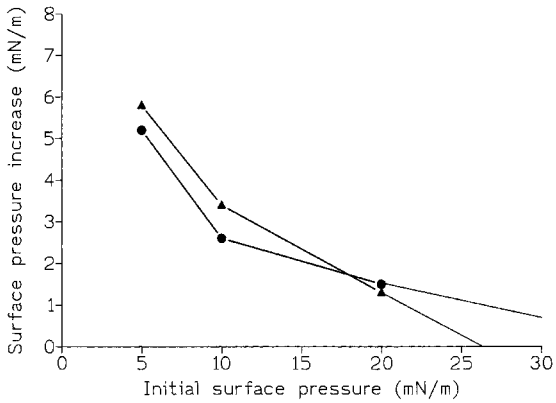


Fig. 3. Surface pressure increase after injection of peptide in the sub-phase of monolayers of DPPC and DPPG spread at different initial surface pressures (peptide concentration in the sub-phase 4 μM). ▲, DPPC; ●, DPPG.

the electrostatic repulsion between the charged monolayer molecules [15]. As surface potential of DPPG monolayers is very high and repulsion between polar heads results in an expansion of the monolayer, the presence in the media of peptide molecules with a positive charge could neutralize, partly, these repulsion forces and promote smaller increases in surface pressure. The values here described are in the same range than those given in the literature for peptide sequences of a similar size [16]. These data indicate that the peptide is able to penetrate softly into these monolayers from Fig. 3 values, a limiting surface pressure of about 25 mN m^{-1} could be derived; extrapolation up to this value, for DPPG monolayers, according to the spreading pressure of the peptide, has no physicochemical meaning.

3.3.1. Compression isotherms

Pressure–area isotherm of pure peptide is shown in Fig. 4. The monolayer has no differentiated phase changes, although at low surface pressures the slope can be associated to a gas state. Up to 5 mN m^{-1}, slope increases and the extrapolated area/molecule is consistent with the area of a β-sheet (ca. 0.6 nm^2 per molecule), that would agree with the insertion of the amino terminal polar aminoacids in the water subphase. Collapse is reached at 25.3 mN m^{-1} of surface pressure at an area molecule around 0.3 nm^2. All these values are in agreement although slightly lower, to those described for the parent peptide (SIKVAV, where no polar aminoacids had been incorporated or a longer sequence where tyrosine residues had been incorporated) [17,18].

Elasticity values were calculated assuming that for an insoluble monolayer the equilibrium elasticity is related to its compressibility C_s and is usually defined as its reciprocal:

$$C_S^{-1} = -A\,[\delta\pi/\delta A]_T, \tag{3}$$

where A is the area/molecule at the indicated surface pressure and π is the corresponding surface pressure. The limiting area is determined at the calculated point of minimum compressibility.

This value can be used to characterize the phase state of a monolayer, according to [19]. High C_S^{-1} values correspond to low in-plane elasticity among packed lipids forming a monolayer. For liquid expanded films, elasticity ranges from 12.5 to 50 mN m^{-1}, while for the liquid condensed phase, ranges from 100 to 250 mN m^{-1}. Inset of Fig. 4 represents these values as a function of the area–molecule. According to the values calculated for elasticity this monolayer is in liquid expanded state all over the compression process reaching directly collapse.

3.3.2. Compression isotherms of mixed monolayers

To better determine the interactions between lipids and peptide, the miscibility of both components was studied at different molar compositions. The physicochemical behavior of these mixtures was controlled through the compression isotherms profiles (area/molecule values) of mixed monolayers. Thermodynamic parameters involved in the mixing process (ΔG_M^{Ex}, α, ΔH, and PF), have been calculated from the area per molecule values both for pure and mixed monolayers.

Miscibility trends are clearly appreciated on the graphics of Fig. 5.

High area/molecule values for mixed monolayers composed of 60 and 80% molar of DPPC indicate the presence of strong repulsive forces between the two types of molecules. Applying Eqs. (4)–(7), energies involved in this process can be quantified as follows [20–22]:

$$\Delta G_M^{EX} = \int_{\pi\to 0}^{\pi} A_{1,2}\,d\pi - X_1 \int_{\pi\to 0}^{\pi} A_1\,d\pi - X_2 \int_{\pi\to 0}^{\pi} A_2\,d\pi \tag{4}$$

$$\alpha = \frac{\Delta G}{RT(x_1 x_2^2 + x_2 x_1^2)} \tag{5}$$

$$\Delta H = \frac{RT\alpha}{z} \tag{6}$$

$$PF = \frac{0.907 A_{c,m}}{A_m} \tag{7}$$

where $A_{1,2}$ is the mean area/molecule in the mixed film, A_1 and A_2 are the areas per molecule in the pure films and π is the surface pressure in mN m^{-1}. Numerical data were transferred from the informatic program of the Langmuir balance to another program that calculates the area under the

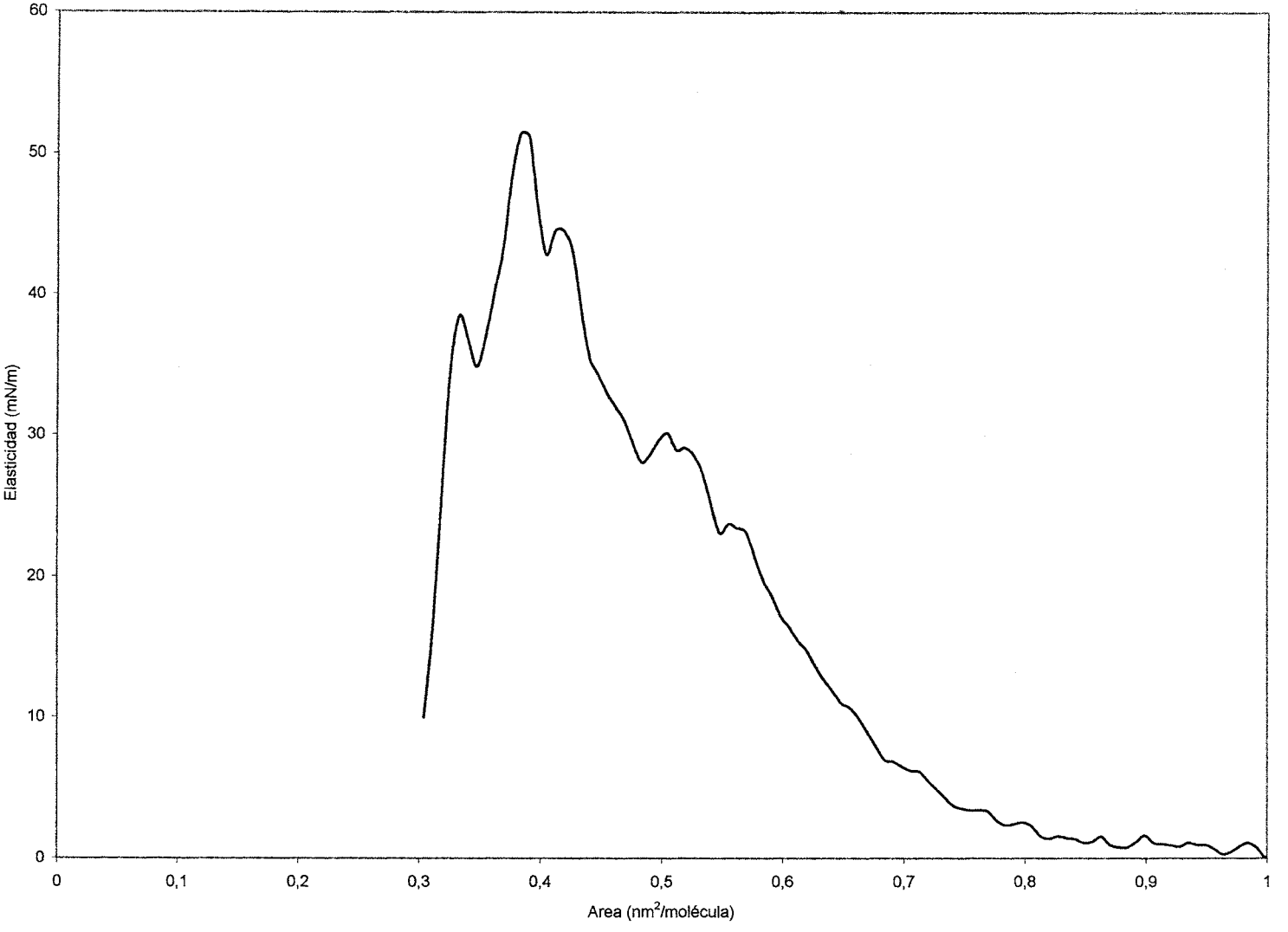


Fig. 4. Compression isotherm of a GESIKVAVS monolayer spread on a PBS subphase at pH 7.4. Inset: Elasticity of the monolayer vs. area/molecule.

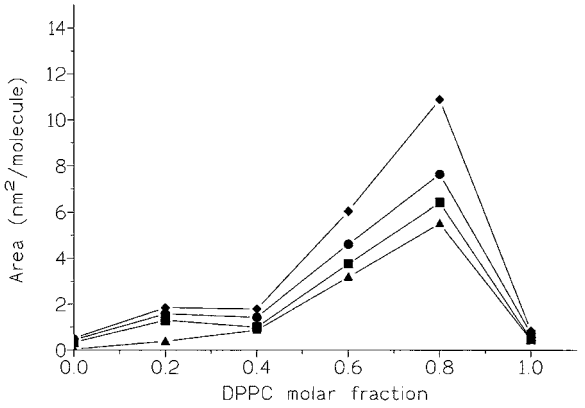


Fig. 5. Area–molecule values calculated for DPPC/peptide mixed monolayers of variable molar composition.

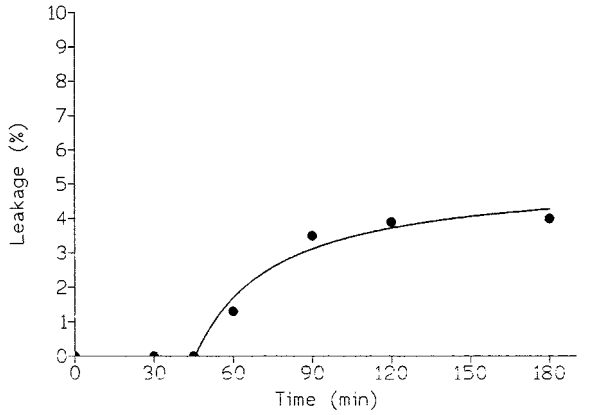


Fig. 6. CF leakage induced by the presence of peptide in the incubation media. Peptide concentration 58 μM.

isotherm at a fixed pressure according to the mathematical method of Simpson.

To calculate the coordination number (Z), the model of Quiquenden and Tam was followed [23], considering that in a closely packed monolayer (collapse),each molecule is surrounded by six neighbors. For lower pressures, Eq. (4) was applied to calculate the packing fraction (PF). This value was used to obtain the corresponding Z, according to the equivalence given by Quickenden and Tam, where $A_{c,m}$ is the area/molecule of the mixture at the collapse point and A_m the area/molecule of the mixture at the pressure studied.

In Table 2 the thermodynamic values calculated as a function of the molar fraction are reported. Taking as reference RT value (2443.14 J mol^{-1}), energies involved in the mixing process at X_{DPPC} 0.6 and 0.8 are well above this figure and indicate the presence of strong interactions. Moreover, the positive sign in the area molecule calculations corresponds to an expansive effect generated when peptide chains are inserted in between the acyl phospholipid ones. Due to this process the cross-sectional area increases strongly because amino acid lateral chains create irregularities or defaults in the ordered packing of DPPC acyl chains disorganizing the initially well packed structure of pure DPPC molecules.

3.3.3. *Leakage of carboxyfluorescein*

After incubation of the peptide (58 μM) with DPPC vesicles loaded with CF, latency values decreased slightly with time suggesting a soft destabilizing effect that reached its maximum value 4% at 180 min time (Fig. 6). This low level of interaction is in agreement with the insertion values determined in monolayer experiments (at high surface pressures), and suggests that the association of this peptide with liposomes is compatible with the conservation of their integrity.

Table 2
Thermodynamic parameters of GESIKVAVS/DPPC mixed monolayers

	ΔG_M^{EX} (J mol^{-1})		ΔH (J mol^{-1})	
Surface pressure	Π (mN m^{-1})		Π (mN m^{-1})	
Fractión DPPC	10	20	10	20
0.8	14.130	20.320	44.150	63.490
0.6	5.870	10.160	4.370	21.160
0.4	1.500	2.840	3.140	2.960
0.2	1.340	1.920	2.090	3.000

3.3.4. Microviscosity studies

The insertion of a molecule in a bilayer can disturb the ordered packing of either the acyl chains or the polar heads resulting in changes in the fluidity of the zone involved. In this study two fluorescent probes were used, DPH for the internal core of the bilayer and ANS that interact with the external surface of vesicles. The influence of peptide (in the incubation media), on the microviscosity of bilayers was determined through fluorescence polarization changes of both probes. Results obtained indicate that the interaction appears only at the level of polar heads (ANS probe), and starts to be evident at temperatures above the transition temperature (T_c) of DPPC (up to 41.5 °C) [24]. At these conditions the peptide has a soft fluidifying effect only at the external part of the bilayer. As far as the internal core of bilayer is concerned, polarization/temperature curves recorded for DPH labeled vesicles either with or without peptide were identical as can be appreciated in Fig. 7, thus confirming the soft interaction of this peptide with liposomes. Nevertheless, as DPH-fluorescence intensities (inset Fig. 7) were lower for samples containing peptide than for reference, its insertion till a certain deepness in the bilayer can not be excluded.

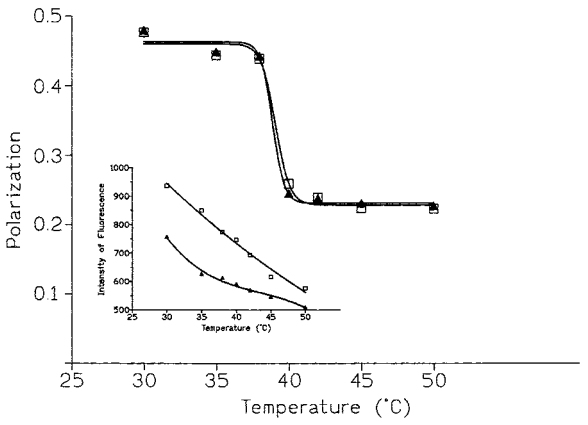


Fig. 7. Polarization of DPH labeled liposomes vs. temperature for samples incubated either with buffer or with peptide. Inset: Fluorescence intensity of samples recorded at increasing temperatures. Peptide concentration 58 μM. ▲, Peptide; □, buffer.

3.3.5. Fusion of vesicles

The leakage of aqueous contents induced by a peptide suggests a certain level of membrane destabilization. This permeation can be due to destabilization of individual vesicles without fusion (pore formation), or to peptide induced fusion. Lipid mixing, monitored by RET between NBD-PE and Rh-PE is a very specific assay because the probes involved do not exchange in the time scale of the experiments, even in aggregated vesicles [25]. As a general trend, after mixing (without fusion), vesicle's spectra show two bands corresponding to the isolated molecules of the fluorophores. A decrease in the band of donor (NBD-PE) accompanied with an increase in the band of the acceptor (Rh-PE) would indicate that molecules were close enough to stablize the transfer of energy and this could only happen if there had been a fusion of bilayers. These studies were carried out with two basic liposomal compositions DPPC and DPPC/DPPG (1:1), containing either (0.6%) NBD-PE, or (0.6%) Rh-PE. Peptide was incubated with these four populations of vesicles at different lipid/peptide relationships. Maximal peptide concentration in the media was 58 μM. Excitation was at 460 nm and emission at 530 nm. Fluorescence spectra of samples did not change with time, neither for cuvettes containing the peptide nor for those used as reference. In all cases the band's size and position were constant indicating that no lipid mixing was induced by addition of peptide to both types of vesicles. This is in agreement with the small interaction detected in the experiments described above.

4. Discussion

The purpose of the present work was to check the stability of liposomes in the presence of a selected peptide sequence. This sequence as stated before was chosen according to the data in the literature as a potential ligand for $\alpha_5\beta_3$ integrin receptors overexpressed in tumor cells. The delivery system designed involved the use of liposomes containing negatively charged phospholipids to better entrap the cationic drug doxorubicin. There are several methods to coat liposomes with pep-

tides, most of them involve a chemical reaction between the free peptide and a reactive group located on the surface of the vesicle, that is why is important to know in advance if the peptide can by itself destabilize the vesicle structure. The results we have obtained indicate that lipid/peptide interactions even at high peptide concentrations do not destabilize the structure of vesicles. Moreover, the small leakage of CF detected in our models would be still lower when using doxorubicin loaded liposomes because of the association of this molecule to PG polar heads. All these data suggest that this sequence can be used to coat liposome's, for targeting applications rendering stable preparations.

References

[1] B. Geiger, A. Bershadsky, R. Pankov, K.M. Yamada, Nat. Rev. Molec. Cell Biol. 2 (2001) 793–799.
[2] Rémi Palmantier, Margret D. George, Steven K. Akiyama, France M. Wolber, Kenneth Olden, John D. Roberts, Cancer Res. 61 (2001) 2445.
[3] Atsushi Utani, Motoyoshi Nomizu, Hiroshi, Matsurra, Kozue Kato, Takashi Kobayashi, Ushio Takeda, Sinichi Aota, Peter K. Nielsen, Hiroshi Shinkai, J. Biol. Chem. 276 (31) (2001) 28779.
[4] N. Fukushima, M. Sakamoto, S. Hirohashi, Mod. Pathol. 14 (5) (2001) 404.
[5] M.L. Ponce, M. Nomizu, H.K. Kleinman, FASEB J. 15 (2001) 1389.
[6] J.H. Chon, E.L. Chaikof, Am. J. Physiol. Cell Physiol. 280 (2001) C1394.
[7] K. Merrett, C.M. Griffith, Y. Deslandes, G. Pleizier, H. Sheardown, J. Biomater. Sci. Polym. Edn. 12 (6) (2001) 647.
[8] A. Massaguer, I. Haro, M.A. Alsina, F. Reig, J. Liposome Res. 11 (1) (2001) 103.
[9] V. Weissig, J. Lasch, A.L. Klibanov, V.P. Torchilin, FEBS Lett. 202 (1) (1986) 86–90.
[10] L.D. Leserman, P. Machy, J. Barbet, in: G. Gregoriadis (Ed.), Liposome Technology, CRC Press, Florida, 1984, pp. 29–40.
[11] B. Frisch, S. Muller, J.P. Briand, M.H.V. Regenmortel, F. Schuber, Eur. J. Immunol. 21 (1991) 185–193.
[12] D. Polo, M.A. Alsina, I. Haro, F. Reig, Langmuir 16 (9) (2000) 4333–4337.
[13] Y. Cajal, F. Rabanal, M.A. Alsina, F. Reig, Biopolymers 38 (1996) 607–612.
[14] A. Ortiz, Y. Cajal, I. Haro, F. Reig, M.A. Alsina, Biopolymers 53 (2000) 455–459.
[15] A.M.G. da Silva, M.I. Visen, Colloids Surf. 144 (1998) 191.
[16] F. Reig, I. Haro, D. Polo, P. Sospedra, M.A. Alsina, J. Colloids Interf. Sci. 239 (2001) 64–69.
[17] N. Almiñana, M.A. Alsina, F. Reig, J. Colloids Interf. Sci., in press.
[18] A. Juvé, L. Rodriguez, I. Haro, M.A. Alsina, F. Reig, Colloids Surf. (A) 142 (1998) 1–7.
[19] X.-M. Li, M.M. Momsen, J.M.M. Smaby, H.L. Brockman, R.E. Brown, Biochemistry 40 (2001) 5954–5963.
[20] F.C. Goodrich, Proceedings of Eleventh International Congress on Surface Activity, Butterwoths, London, 1957.
[21] R.E. Pagano, N.L. Gershfeld, J. Colloids Interf. Sci. 41 (1972) 311–321.
[22] P. Joos, R.A. Demel, Biochim. Biophys. Acta 183 (1969) 447–452.
[23] T.I. Quickenden, G.K. Tam, J. Colloid Interf. Sci. 48 (1974) 382–387.
[24] A. Juvé, P. Sospedra, L. Rodriguez, F. Reig, Bioluminiscence 17 (2002) 266–267.
[25] D. Hoekstra, O.C. Martin, Biochemistry 21 (1982) 6097.