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The covalent coupling of HAV-VP3 (110–121) synthetic peptide to liposomes: physicochemical studies

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Abstract

In this work we have studied the conjugation of the immunogenic peptide sequence (110–121) belonging to the VP3 capsid protein of hepatitis A virus to the surface of preformed liposomes by means of an amide bond between the vesicles and the synthetic peptide.

The surface activity of the conjugate at air/water interface was determined. Moreover, the interaction of the conjugate with lipids was also studied recording the pressure increases produced after the injection of the liposome–peptide preparation under dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and stearylamine (SA) monolayers at different initial surface pressures. As expected, due to the negative net charge of the liposome–peptide complex, the higher interaction was found with positive charge monolayers (SA). However, the conjugate was also able to incorporate to zwitterionic and anionic lipids. This behaviour was also confirmed performing compression isotherms of monolayers of these lipids spread on subphases containing the conjugate.

These results suggest that the coupling of VP3 (110–121) to liposomes does not influence its ability to interact with membrane lipids such as DPPC and DPPG. Then it can be assumed that its immunogenicity will be preserved or even increased after this modification. All these results are also useful in the preparation of liposome-based synthetic peptide vaccines.
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1. Introduction

Inactivated hepatitis A vaccines are already licensed in many countries (Peetermans, 1992; Mengiardi et al., 1995). Although they are highly immunogenic, the cost involved in their production does not make them viable for vaccination campaigns in developing countries. The use of peptide fragments to generate antibodies able to recognize and neutralize viral particles could be a valuable alternative to conventional vaccines: they are safe, they can be designed to induce well-defined immunoresponses, and they can be synthesised in large quantities in high purity (Arnon, 1991).

However, single proteins or peptide-based vaccines usually require more than a single administration to induce protective response due to their relatively poor immunogenicity compared to classic killed or live attenuated vaccines. Mainly regarding its application in developing countries, any system allowing the administration of vaccines in a few or single doses will be positively considered. While various systems have

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been studied for potential application in vaccine delivery, liposomes have been shown to have some advantages as vaccine carriers because of their immunoadjuvant properties and their capacity to transport a variety of therapeutic agents (Galvain et al., 1999; Alving, 1987). As a result, a variety of protein and peptide antigens have been successfully formulated in liposomes (Waassef et al., 1994; Alving et al., 1995).

The flexibility and versatility of liposomal formulation methods make liposomes an ideal system for probing immunological responses. They may contain soluble antigens in their internal aqueous volume or amphipathic antigens incorporated in the bilayer or covalently coupled to phospholipids contained in the liposomal bilayer (Boeckler et al., 1996). To generate such liposomes, the peptide can be coupled to the lipid and the lipopeptides can be subsequently incorporated into a liposome (De Frees et al., 1996; Yagi et al., 2000) or alternatively, the liposome can be formed first and the peptide attached to head groups exposed on the exterior surface (Muñoz et al., 1998; Schelte et al., 2000; Boeckler et al., 1999).

Using this later strategy, in the present work we have studied the conjugation of the inmunogenic peptide sequence (110–121): FWRGDLVFDFQV belonging to the VP3 capsid protein of hepatitis A virus (Perez et al., 1995) to the surface of preformed vesicles by an amide bond between the liposome and the synthetic peptide. VP3 (110–121) peptide sequence was recognised by human convalescent sera and a high immunogenicity when administered to rabbits entrapped into neutral liposomes was detected (Bosch et al., 1998).

On the other hand, in order to use liposomes as an effective tool in biology, it is first necessary to understand the ways in which they can interact or associate with biomembranes (Macquaire et al., 1992). An understanding of the biophysical nature of the interactions of synthetic peptides with model membranes will help in understanding the molecular properties involved in the association of peptides with membranes in cells. It has been found that electrostatic forces play an important role in the interaction of HAV with cells in the endocytosis process (Bishop and Anderson, 1997). In previous studies the free peptide VP3 (110–121) showed a different pattern of interaction in front of lipid monolayers composed of lipids with different charge (Sospedra et al., 1999). The main objective of the present paper was to analyse the experimental data concerning the interaction of lipids of different nature with the VP3 (110–121) peptide sequence but coupled to liposomes to get more insights into the degree of peptides association to liposomes and the influence of this process in the peptide and bilayer stability. Both are important points regarding the immunogenic results of the final administration form.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol (DPPG), egg phosphatidylcholine (PC), cholesterol (CHOL) glutaric acid and stearylamine (SA) were from Sigma.

Quantitative phosphate analysis was carried out following the method described in McClare C.W.F., 1971.

Chloroform and methanol (pro analisi) and *N*-(3dimethylaminopropyl)-*N*′-ethylcarbodiimide chloride (EDC) were from Fluka, UK. Water was distilled twice and passed through a MilliQ filtration system. The experiments were carried out in 0.05 M phosphate-buffered solution (PBS) (sodium chloride 0.28%, sodium phosphate monobasic dihydrate 0.23% and sodium phosphate dibasic 1.15%) of pH 7.4 and 0.01 M borate solution (boric acid 2.474 $g\,l^{-1}$ and sodium hydroxide 0.2 M 100 ml) of pH 6.0, 6.7 and 7.5.

Trifluoracetic acid (TFA) and *N*-hydroxysulfosuccinimide (NHS) were from Fluka, Switzerland.

2.2. Methods

2.2.1. Liposome preparation

Small unilamellar vesicles (SUV liposomes) were prepared with (PC:Chol:NG-PE (9:10:1, ratio molar)) *N*-glutarylphosphatidylethanoamine (NGPE) was a synthetic derivative of DPPE (Kung and Redeman, 1986).

The procedure of preparation has been previously described (Garcia et al., 1996). Briefly, phospholipids were dissolved in chloroform in a round bottom flask and dried in a rotatory evaporator to from a thin film on the flask. The film was hydrated with a borate solution to form multilamellar vesicles (MLV). MLV were

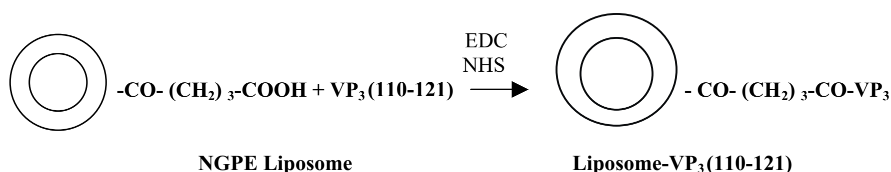

Fig. 1. Strategy for preparation of liposome-linked VP3 (110–121) peptide.

sonicated (Vibracel VC) at 300 W for 2 min. This step was repeated eight times.

2.2.2. Covalent coupling of VP3 (110–121) synthetic peptide to liposomes

Coupling of VP3 (110–121) peptide to preformed vesicles was established by means of an amide bond as shown in Fig. 1.

To obtain an amide bond between preformed SUV-liposomes containing the NGPE and the VP3 (110–121) peptide, liposomes were activated by NHS and EDC at room temperature for 1 h (Muñoz et al., 1998). Then, an equimolar amount of peptide dissolved in 3% DMSO/borate buffer 0.01 M pH 6 was added. Final pH of incubation media was adjusted to 7.5 with NaOH 0.2 M and the conjugation reaction was carried out overnight at 4 °C. Free peptide was separated from liposomes by dialysis against borate solution pH 7.5.

2.2.3. Characterisation of liposome–peptide conjugates

Vesicles size was determined prior and after the conjugation to peptide by measurement of the sample diffusion coefficient by photon correlation spectroscopy (Table 1). Phospholipid content was analysed as previously described (McClare, 1971).

Estimation of total liposome-associated peptide was achieved by quantitative amino acid analyses. The

Table 1
Vesicle size and phospholipid content of liposome–peptide conjugate

Liposome	
Size (nm)	84.76 ± 7.64
Polydispersity	0.19 ± 0.07
Liposome–peptide	
Size (nm)	154.63 ± 50.69
Polydispersity	0.24 ± 0.10
Phospholipid content (mg ml^{-1})	2.18 ± 0.59

analyses were carried out in a Pico-Tag system (Waters, Stockport, UK). Samples were hydrolysed in 6 M HCl at 110 °C over 24 h. Traces of non-covalent coupled peptide (adsorbed on the vesicle surface) were determined by detection of free peptide by analytical HPLC after disruption of liposomes with methanol. These analyses were performed on a Spherisorb ODS (10 μm) column eluted with acetonitrile/water (0.05% TFA) mixtures. Conditions used for liposome samples were 3 min isocratic 75% of A (acetonitrile/H_2O (80/20)), and B (25% H_2O) followed by a gradient from 75% of A to 100% of A during 30 min. To obtain the percentage of covalent yield (referred to the initial peptide added) the non-specifically adsorbed peptide, determined as described above, was subtracted from the total peptide that was present in the liposome preparation, that was determined by amino acid analysis as previously described.

2.2.4. Fluorescence study

Fluorescence experiments were carried out on a Perkin-Elmer (Beaconsfield, bucks, UK) spectofluorimeter LS 50. Cells of 1 cm optical path were used. Fluorescence spectra were measured at room temperature in 5 mM Hepes buffer pH 7.4.

VP3 (110–121) contains Phe and Trp amino acids which may contribute to the fluorescence spectrum. However, upon excitation at 295 nm only the Trp emission will be measured, because under these experimental conditions Phe will not be excited and, consequently, its small quantum yield will not be observed.

Peptide samples were excited at 295 nm with a slit width of 12 nm for the excitation and emission beams. Emission spectra were recorded from 300 to 450 nm.

2.2.5. Studies in monolayers

Measurements were performed on a NIMA Langmuir film balance and through (601 BAM) equipped with a Wilhelmy plate.

2.2.5.1. Surface activity of the liposome–peptide conjugate. The surface activity of the liposome–peptide conjugate measurements were performed in a Teflon trough, cylindrical in shape and with 70 ml of capacity. Surface tension was measured as a function of time by the Wilhelmy plate method, using a platinum plate.

The subphase was PBS pH = 7.4. Increasing volumes of concentrated solutions of liposome–peptide conjugate were injected below the surface through an injection septum and pressure increases were recorded for 60 min.

2.2.5.2. Insertion of liposomal peptides into monolayers. The same trough described above was used in these studies. Stock solutions of DPPC, DPPG and SA in chloroform or chloroform/methanol (3:1) in SA case, were spread at the air/water interface using a Hamilton syringe, to attain 2.5, 5, 10, 20 or 32 mN m^{-1} of initial surface pressure. After a stabilisation period of 15 min, liposomal preparation, at a slightly lower liposomal peptide concentration than that corresponding to the saturation, was injected into the subphase and the increase in surface pressure recorded for 60 min.

2.2.5.3. Compression isotherms. In this case a trough of area 500 cm^2 and volume 250 ml was used. Lipid monolayers were formed on a PBS subphase, and after allowing 15 min for solvent evaporation, films were compressed at a rate of 100 cm^2 min^{-1}. The same process was repeated with a subphase containing the liposome–peptide conjugate at a 0.9 μM concentration of peptide, but after spreading the monolayer 30 min were allowed for the adsorption of the conjugate to the monolayer.

All experiments were performed in triplicate at a temperature of 21 ± 1 °C.

3. Results and discussion

3.1. Characterisation of liposomal constructs

As shown in Table 1 liposome–peptide conjugates were characterised by measurement of vesicles size and phospholipid content.

Our results indicate similar results for the three preparations both in size and phospholipid content determinations. Moreover the liposomes were uniformly distributed in size, thus polydispersity values were in all cases lower than 0.25.

The increase in size of liposomes upon coupling of the peptide can be explained by an aggregation process that is favoured by the peptide located in the surface vesicles. Similar results have been obtained previously working with other HAV-related peptides (Muñoz et al., 1998).

The amount of peptide associated with liposome-preparations was 0.54±0.19 mg peptide per milligram phospholipid and covalent yield obtained which is referred to the total amount of added peptide resulted 62.6 ± 26.8. Having in mind that the NGPE derivate is partly facing the inside of the vesicles and remains unavailable to the peptide, we consider the covalent yield obtained highly satisfactory.

3.2. Fluorescence

The fluorescence spectrum of Trp is a very sensitive indicator of the microenvironment of the amino acid. VP3 (110–121) has a single Trp at position 111, this is at the second position from the amino terminus.

The fluorescence properties of Trp depend on its environment and therefore changes in fluorescence intensity and emission maximum give information on the location of the amino acid in the lipid bilayer of SUV liposomes.

In Fig. 2 are shown the Trp fluorescence spectra recorded for the VP3 free peptide and for the

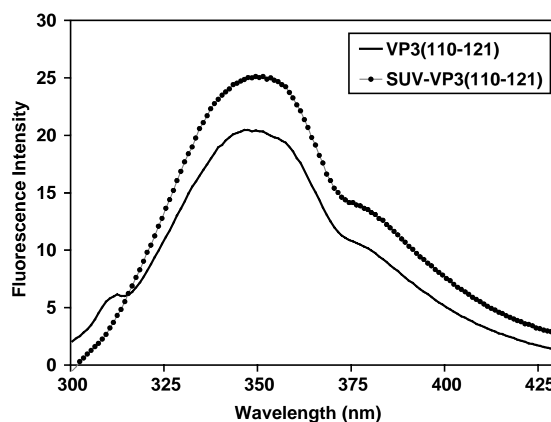

Fig. 2. Fluorescence spectra of VP3 (110–121) and SUV liposome–VP3 (110–121) conjugate. Spectra were recorded at 20 °C at a peptide concentration of 3 μM. Excitation wavelength is 280 nm.

liposome–VP3 peptide conjugate at a peptide concentration of 3 μM after excitation at 295 nm. The maximum of emission obtained in both cases are almost identical and centred in 350 nm indicating a high degree of exposure of the Trp residue to the bulk aqueous phase. Moreover an slight increase in fluorescence intensity accompanies binding VP3 (110–121) to SUV-liposomes. This result could indicate a more hydrophobic environment for Trp, reflecting some degree of insertion of the peptide into the hydrophobic core of the bilayer. However, a discrete conformational change of the peptide seems more plausible. This result can thus be interpreted as being due to the partially buried character for Trp when covalently coupled to liposomes, due to the high propensity as previously described (Vea et al., 1998) to form β-structures, that could favour an aggregated state.

Taken together, these results indicate that the covalent linkage of VP3(110–121) peptide to preformed liposomes, by means of an amide bond between the NGPE phospholipid and the amino acid N-terminal (Phe) of the VP3 peptide, do not significantly alter the Trp environment.

However, as claimed by other authors, the bioactive conformation of a peptide is generally evoked by conformational changes induced through its interactions with biomembranes (Woolley and Deber, 1987; Ono et al., 1990).

In this sense the transconformation triggered by the conjugation of the VP3 peptide to the surface of liposomes could be a characteristic feature required for its recognition because peptide activity is in general related to its conformation at the binding site.

3.3. Surface activity

The surface activity of the peptide coupled to liposomes was determined by injecting different volumes of liposome–peptide preparations into the PBS surface and recording the surface pressures achieved. The experimental curves were used to determine the peptide concentration to be employed in the kinetics at constant area. Liposomes without peptide were used as control, the pressure increase being less than 10% of the maximum measured.

The conjugate showed a gradual adsorption to the air/water interface at low concentrations, at higher concentrations the adsorption was faster and the equilibrium was reached earlier. Fig. 3 represents pressure increases obtained at 60 min (in all cases the equilibrium had been reached) for the different concentrations assayed. It can be observed that around 3 μM of conjugate concentration the pressure increase is practically constant, suggesting that saturation was reached at the air/water interface. This saturation concentration was lower than that obtained when a longer VP3 sequence was studied (Garcia et al., 1996).

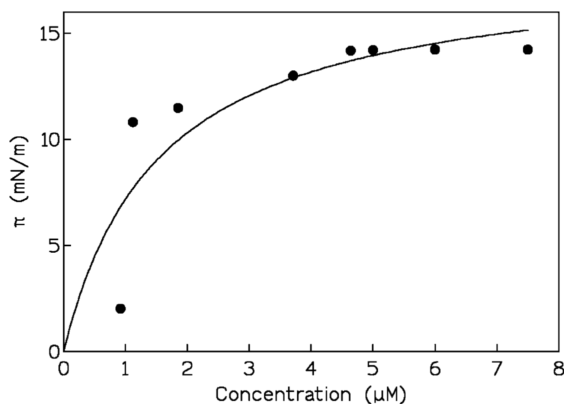

Fig. 3. Effect of the conjugate concentration on surface pressure. Surface pressure values were determined after 60 min of conjugate injection into the subphase.

Compared with the values obtained in similar conditions with the free peptide (Sospedra et al., 1999), the pressure increase values were around 20–25% lower. This lower surface activity could be explained by several factors. On the other hand, for topological reasons only a fraction of the liposomal peptide could engage in the interactions with the monolayer.

3.4. Insertion of liposome–peptide conjugate to lipid monolayers

The ability of the VP3 (110–121) peptide coupled to SUV liposomes to insert into lipid monolayers was studied by injecting a lower concentration than the saturation one (0.92 μM) beneath DPPC, DPPG and SA monolayers spread at 2.5, 5, 10, 20 and 32 $mN\,m^{-1}$ of initial surface pressure and recording the changes in the surface pressure increase for 60 min. In all the monolayers assayed, pressure increased slowly, as can be seen in Fig. 4 for kinetics corresponding to DPPC monolayers. In DPPC and DPPG monolayers the pressure increases achieved had very different values

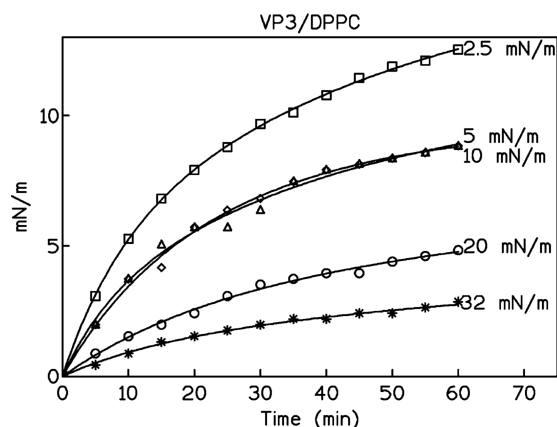

Fig. 4. Surface pressure increases vs. time for DPPC monolayers.

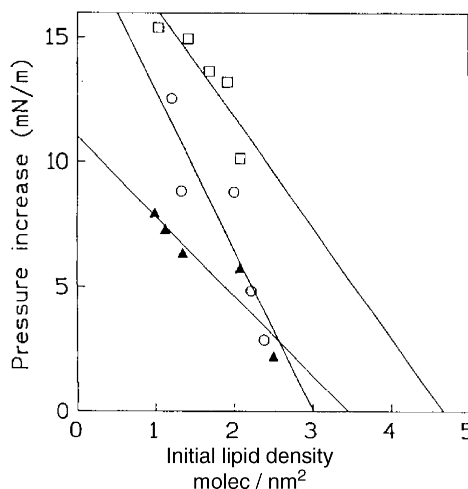

Fig. 5. Surface pressure increase plotted as a function of the initial density of lipids. (□) SA, (○) DPPC and (▲) DPPG.

depending on the initial surface pressure, however, for SA the pressure increases measured were very similar in all cases. Moreover, when working with SA monolayers the pressure increases were higher than that obtained with the other lipid monolayers (DPPC and DPPG) (Table 2). In order to have a better understanding of these interactions (Fig. 5) we have plotted the pressure increase in front of lipid density (Maget-Dana, 1999). It can be deduced from this figure that the conjugate incorporates in a higher extent to SA monolayers producing higher pressure increases in all cases. Its behaviour in front of DPPC and DPPG is more difficult to interpret. Even though the peptide presents one negative net charge it was able to incorporate in an anionic lipid such is DPPG. It also can be observed that the exclusion monolayer density is even a little lower for the zwitterionic lipid DPPC. All this suggest that hydrophobic interactions have also an important role in the incorporation of the conjugate into the monolayer, that can be potentiated by the liposome moiety of the complex.

Table 2
Parameters obtained of experimental data from penetration kinetics curves for amide coupled peptide to SUV liposomes

	$\Delta\pi_0$ (mN m^{-1})
DPPC π_0 (mN m^{-1})	
2.5	12.54
5	8.84
10	8.80
20	4.84
32	2.86
DPPG π_0 (mN m^{-1})	
2.5	7.92
5	7.26
10	6.32
20	5.72
32	2.20
SA π_0 (mN m^{-1})	
2.5	15.40
5	14.95
10	13.64
20	13.20
32	10.12

3.5. Compression isotherms

The interaction of the conjugate with lipid monolayers was also studied through compression isotherms. Fig. 6a–c shows the isotherms obtained after compressing DPPC, DPPG or SA films spread on PBS subphase or on PBS containing the conjugate at a concentration of 0.92 μM.

In all cases the presence of the conjugate produced an increase in the values of area/molecule. The phase transition present in the isotherms of lipids spread in absence of conjugate disappeared when the conjugate was present in the subphase. The higher effect was found in SA monolayers, with a maximum increase in area/molecule and important modification in the shape and collapse pressure. From these results one can speculate that the conjugate is incorporated to

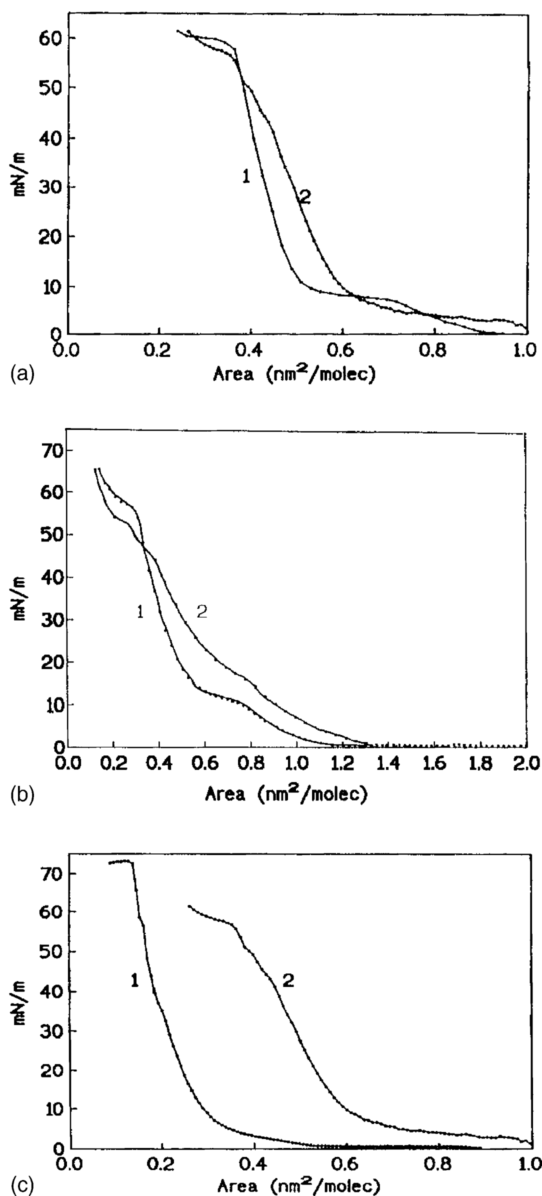

Fig. 6. Compression isotherms of lipid monolayers spread on: (1) PBS subphase, (2) conjugate 0.9 μM solution subphase. (a) DPPC, (b) DPPG and (c) SA.

the monolayer, because if the conjugate had been excluded from the film during compression, then the isotherms of the films in presence of conjugate would have converged with those of the pure lipids. This convergence is only seen at high pressures (near the collapse) for DPPC and DPPG monolayers. These results are in agreement with those found in the kinetic assays (Table 2), where the higher interaction was found with SA. Compared with the parent peptide (Sospedra et al., 1999), the conjugate had a higher interaction with SA. The low degree of interaction with DPPG monolayers, also found in kinetic and isotherm studies, could probably be due to the contribution of repulsive forces established between the negative net charges of both the lipid and the peptide.

To sum up, conjugation of VP3 (110–121) to liposomes decreased the surface activity compared with the free peptide even though that the VP3 (110–121) coupled to the liposome retains the main conformation obtained for the free peptide in a membrane environment (Perez et al., 1998). However, this lower surface activity does not imply a reduction in the interaction of the conjugate with monolayers composed of lipids of different characteristics.

Electrostatic interactions, as was expected, have an important role, as it was clearly seen in SA monolayers. However, results reported in this work show that the peptide liposome conjugate binds to lipids with different net charge (zwitterionic, anionic and cationic) thus indicating that the binding have also a significant hydrophobic component. It can be expected that VP3 (110–121) coupled to liposomes can interact with biological cell membranes containing DPPC and DPPG. Also, its high interaction with SA can be useful in order to use this lipid in liposomal preparations containing the VP3 (110–121) peptide sequence. The results reported here can be useful in the preparation of liposome based synthetic peptide vaccines.

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