Interaction Mechanisms between a Homologous Series of Tripodal Cationic Peptides and Lipid Bilayer Membranes

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Introduction

An increasing number of natural and synthetic peptides with the ability to translocate the plasma membrane are being investigated for their application as drug delivery agents. These cell-penetrating peptides (CPP) are recognized by their capability to enter cells via a non-endocytic and receptor-transporter exclusive pathway [1]. Peptide-membrane interactions and membrane translocation are key properties for intracellular delivery. Although exact mechanisms underlying these interactions remain unknown; they arise from the combined effect of hydrophobic interactions of the peptides with the hydrocarbon lipid chain region and electrostatic attractions with oppositely charged lipids. Many CPPs, due to their cationic nature, can interact strongly with anionic cell membranes [2].

Wender *et al.* [3] have described the significance of positive residues in the membrane penetration ability of Tat (trans-activating transcription factor) peptides. Arginine residues weakly bind to phosphate groups of the lipids that form the framework of the cytoplasmic membrane, neutralizing the charge and causing them to partition into the glycerol region of the lipid headgroups. This is followed by penetration into the hydrophobic bilayer and pore formation, Chan *et al* [4]. Decreasing two arginine moieties can reduce the translocating ability by 75% followed by loss of activity of CPPs , Tung *et al* [5].

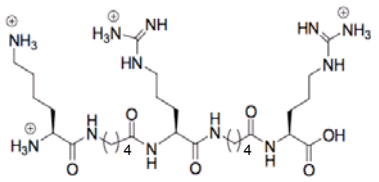
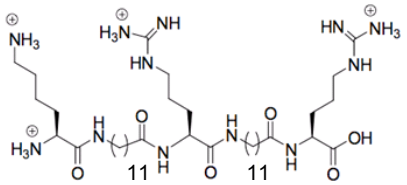
We have developed a series of *de novo* tripodal peptides based on the SRC binding domain of tyrosine kinase responsible for phosphorylation. These peptides possess both cationic and lipophilic properties – two arginine residues and a lysine residue coupled through two C_n alkyl linkages (Table 1). LPA- C_4 and LPA-C_{11} were added to liposomes composed of zwitterionic DPPC, and anionic DPPG (one negative charge on the phosphate group) and DPPS (one positive and two negative charges), to determine the role of the hydrophobic peptide linkage and bilayer charge on peptide adsorption and bilayer disruption. Differential scanning calorimetry (DSC) was used to characterize the influence of the peptides on the thermodynamic phase behavior of the lipid mixtures. Our results correlate with the cellular uptake studies conducted by Ye *et al.*, [6] and confirm an increase in membrane localization and anchoring of peptides with increased C_n spacing between the amino acid residues.

Experimental

Peptide Synthesis

The peptides (Table 1) were synthesized according to solid phase Fmoc strategy (Figure 1), by utilizing Fmoc-Arg(Pbf)-Wang resin as an initiating amino acid and Fmoc-L-amino acids- arginine and lysine as building blocks. Synthesis involved deprotection of Arg-attached Wang resin, followed by coupling reaction with $FmocNH(CH_2)_n$, Fmoc-Lys(Boc)-OH.Lys, and Fmoc-Arg(Pbf)-OH. HBTU and NMM in DMF were used as coupling and

Table 1. Characteristics of synthesized peptides.

Peptide	Structure	Mol. wt.
LPA-C_4		657
LPA-C_{11}		853

activating agents respectively. Fmoc deprotection was conducted using a mixture of piperidine in DMF (20%). with an alkyl chain linker. After peptide synthesis, resin was treated with piperidine (20% in DMF). A

mixture of TFA, anisole and water (90:5) was used for side chain deprotection of amino acids and cleavage of the synthesized peptide from the resin. Crude peptides were then purified by reverse-phase HPLC. Peptide stock solutions were prepared at different concentrations in sterile, deionized ultra-filtered water at pH 7.

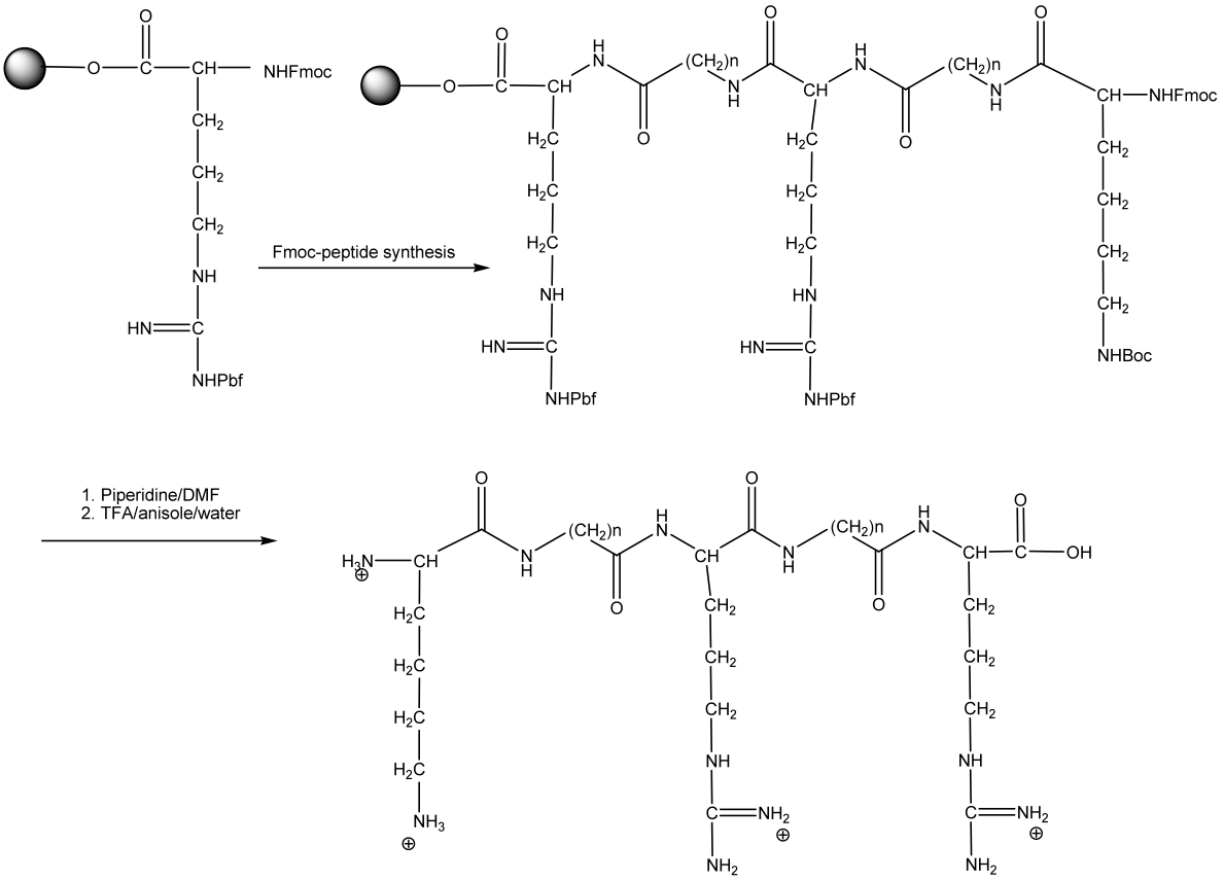


Figure 1. Schematic of peptide synthesis by Fmoc strategy.

Liposome Preparation and Phase Behavior

Stock solutions of DPPC were prepared in chloroform, and DPPC/DPPG and DPPC/DPPS were prepared in chloroform/methanol (1:1). Aliquots of the stock solutions were dried under a stream of nitrogen followed by vacuum drying overnight. The dried films were hydrated with peptide solutions at different concentrations. For differential scanning calorimetry (DSC), the hydrated samples were prepared at 30 mM lipid concentration and sonicated for 15 min before analysis. Samples (20µL) were added to aluminum hermetic DSC pans. All samples were heated and cooled sequentially for three cycles at a rate of 1°C/min between 25 and 60°C.

Results and Discussion

Synthesized peptides were analyzed by using surface-enhanced laser desorption/ionization (SELDI) technique. Purified peptides were cocrystallized with UV absorbing sinnapic acid and vaporized with pulsed-UV laser beam. The proteomic spectra obtained showed a sharp peak for the peptide.

DSC was used to study the thermotropic behavior of DPPC, DPPC/DPPG and DPPC/DPPS vesicles in the presence of peptides. Peptides were added at concentrations of 15, 20 and 30mol% relative to lipid, during the formation of liposomes. During heating, DPPC liposomes (control sample) exhibited a broad low enthalpy pretransition at 35^{o}C and a sharp main transition at 41^{o}C (Figure 2). The pretransition refers to gel to rippled gel transition and the main transition describes a shift from a rippled gel to a fluid phase. Addition of LPA-C_4 at different concentrations did not significantly alter the phase transition behavior of DPPC lipid bilayers. In contrast, the more lipophilic LPA-C_{11} eliminated the pretransition, and reduced and broadened the main transition.

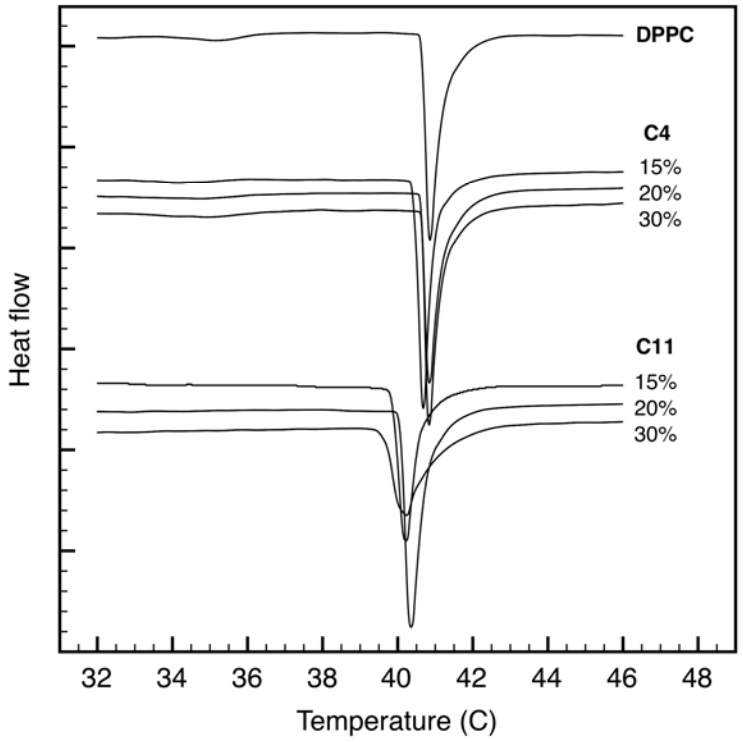


Figure 2. DSC thermograph representing the effects of C_4 and C_{11} peptides on DPPC bilayers at different concentrations (15, 20 , 30 mol% lipid basis).

Figure 3 is a representative DSC thermograph of LPA- C_4 and LPA- C_{11} with an anionic lipid mixture, DPPC/DPPG (85/15 molar ratio), with 15 and 30mol% peptides (lipid basis). For the control sample, in the absence of peptides, only one main transition peak was observed indicating miscibility of the phospholipids within the bilayer. Increasing LPA- C_{11} concentration (30 mol%) caused broadening of the main transition peaks, decreasing its melting temperature by 1.85°C. Addition of 15 mol% of C_4 showed a substantial widening of the main transition peak and a further increase in melting temperature was observed at 30 mol% C_4 suggesting greater interaction between the cationic peptides and anionic lipid bilayer.

Lipid melting temperature and enthalpy at the main transition, full peak width at a half maximum height (FWHM) and cooperative lipid melting unit (C.U.) for all samples are summarized in Table 2. The most significant decrease in melting temperature was observed when 30 mol% LPA-C_4 and LPA-C_{11} interacted with DPPS/DPPC (85/15) bilayers (ΔT_m = -2.12 and -3.51°C, respectively). In general, with increasing peptide concentration, a pronounced change in T_m was observed with a reduction in the number of cooperative units in the bilayer. In general, LPA-C_{11} had a greater impact on bilayer phase behavior than LPA-C_4 due to its greater lipophilicity. Our results indicate that electrostatic interactions play a key role in governing peptide-bilayer interactions; however, the peptide must be sufficiently lipophilic to induce bilayer disruption.

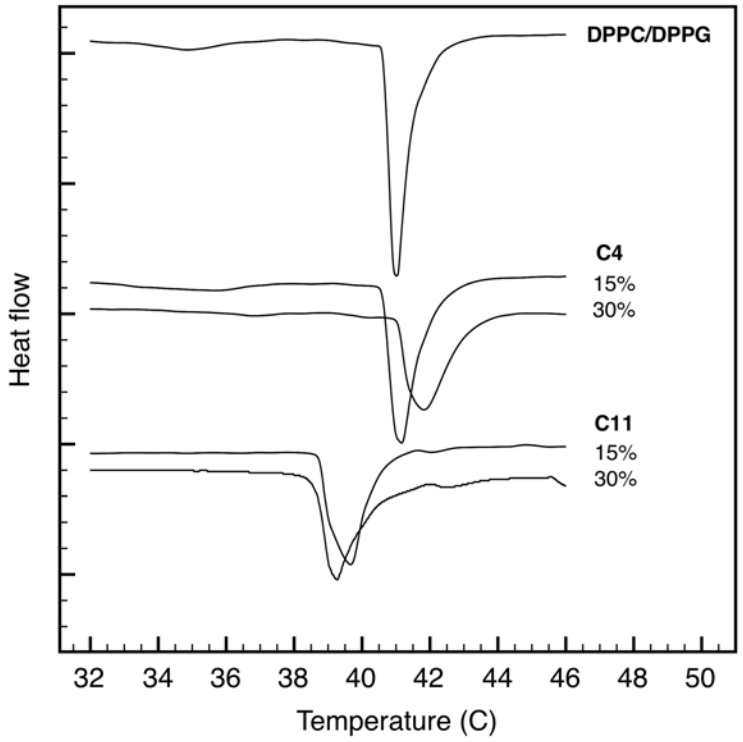


Figure 3. DSC thermograph of DPPC/DPPG (85/15) bilayers at 15 and 30 mol% peptides.

These results correlate with the cellular uptake studies of the peptides in BT-20 cells conducted by Ye *et al.* where LPA-C_{11} was internalized (non-endocytotically) while LPA-C_4 was not. This was due to the lipophilicity of LPA-C_{11}, which, in model cell membranes, led to bilayer disruption. In addition to these studies, we are investigating leakage of entrapped carboxyfluorescein from the liposome-peptide systems by fluorescence spectroscopy. We are also monitoring the detailed structural changes occurring in the liposomes undergoing interaction with peptides using cryogenic transmission electron microscopy (cryo-TEM). By combining these techniques, we seek a detailed understanding of the effects on synthetic peptides on bilayer phase behavior, permeability, and structure.

Table 2. Thermotropic parameters for the peptide-lipid interactions determined by DSC.

Lipid[a]	Peptide	T_m (°C)[b]	ΔT_m (°C)[c]	FWHM (°C)[d]	□H (kJ/mol)[e]	C.U. (# of lipids)[f]
			15 mol% peptide			
DPPC	control	40.8	0	0.31	25.92	360
	LPA-1	40.6	-0.12	0.30	24.64	388
	LPA-4	40.2	-0.58	0.48	29.27	205
DPPC/DPPG	control	40.9	0	0.50	33.94	171
	LPA-1	41.1	0.19	0.14	29.81	85
	LPA-4	39.5	-1.45	0.95	26.24	115
			20 mol% peptide			
DPPC	LPA-1	40.7	-0.07	0.29	24.30	410
	LPA-4	40.2	-0.53	0.32	23.57	382
			30 mol% peptide			
DPPC	LPA-1	40.8	0.05	0.37	30.59	255
	LPA-4	39.9	-0.82	0.60	27.20	176
DPPC/DPPG	LPA-1	41.8	0.85	1.09	22.09	121
	LPA-4	39.1	-1.85	0.86	27.00	123
DPPC/DPPS	control	43.3	0	1.07	24.71	111
	LPA-1	41.2	-2.12	1.28	20.40	111
	LPA-4	39.8	-3.51	1.26	17.05	134

[a]mixed bilayers at 85 mol% DPPC.
[b]lipid melting temperature at the midpoint of the main phase transition.
[c]change in T_m relative to the control.
[d]full peak width of the main phase transition at half of the maximum peak height.
[e]calorimetric enthalpy of the main phase transition.
[f]cooperative lipid melting unit based on the van't Hoff and calorimetric enthalpies of the main phase transition (C.U. = $\Delta H_{VH}/\Delta H$). ΔH_{VH} is defined as the amount of energy required for each cooperative unit to undergo melting. C.U. was calculated on a DPPC basis.

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