Supporting Information

Novel neutral imidazole-lipophosphoramide for transfection assays

Mathieu Mével[a], Cécile Neveu[a] , Cristine Gonçalves[b], Jean-Jacques Yaouanc[a*], Chantal Pichon[b], Paul-Alain Jaffrès[a,] Patrick Midoux[b]

a- Laboratoire de Chimie Electrochimie Moléculaire Chimie Analytique, Université de Bretagne Occidentale, CNRS UMR 6521, 6 Avenue Le Gorgeu, 29238 Brest, France.

b-Centre de Biophysique Moléculaire, CNRS UPR 4301 affiliated to Inserm and Université d'Orléans, Rue Charles Sadron, 45071 Orléans cedex 2, France

e-mail: jean-jacques.yaouanc@univ-brest.fr ; patrick.midoux@cnrs-orleans.fr

General Methods. Unless otherwise stated, all reagents were purchased from Sigma (St Quentin Fallavier, France). Solvents were freshly distilled on appropriate driers and reactions run under nitrogen atmosphere (CH_2Cl_2 was distilled over P_2O_5, DIPEA was distilled over NaOH). Reactions were purified by chromatography column with silica gel Si 60 (0.040–0.063 nm). Thin layer chromatography was carried out on silica gel 60 F254 (1.1 mm) with spot detection under UV light or through I_2 or $KMnO_4$ oxidation. All compounds were fully characterized by ^{1}H (500 MHz), ^{13}C (100 MHz) and ^{31}P (121.49 MHz) NMR spectroscopy (Bruker AC 300 and Advance DRX 500 spectrometers, Service de RMN, UFR Sciences et Techniques, Université de Bretagne Occidentale,Brest). NMR spectra were obtained in $CDCl_3$ unless otherwise stated. All chemical shifts (δ) and coupling constants are quoted in parts per million (ppm) and Hertz (Hz) respectively. The following abbreviations were used: s for singlet, d doublet, t triplet, q quadruplet, qt quintuplet, m for multiplet. The chemical shifts are calibrated to TMS (δ H 0.00) or residual proton and carbon resonance of the solvent $CDCl_3$ (δ H 7.26 and δ C 77.16). When needed, ^{13}C heteronuclear HMQC and HMBC were used to unambiguously establish structures. Mass spectroscopy analyses were performed by CRMPO (Université de Rennes 1, Rennes-France) by using a MS/MS high resolution Micromass ZABSpecTOF.

Compounds **1**[1], **2**[1], **3**[2] and dioleylphosphite[3] were synthesized according to the reported methods.

[1] Mével, M. ; Breuzard, G. ; Yaouanc, J. J. ; Clément, J. C. ; Lehn, P. ; Pichon, C. ; Jaffrès, P.A. ; Midoux, P. *ChemBioChem* , accepted manuscript.
[2] Le Ny, K. PhD Thesis, Brest, 2004.
[3] Floch, V. ; Le Bolc'h, G. ; Gable-Guillaume, C. ; Le Bris, N. ; Yaouanc, J.J. ; des Abbayes, H. ; Férec, C. ; Clément , J.C. *Eur. J. Med. Chem*. **1998**, *33*, 923-934.

Synthesis of phosphoramidates 1 and 2. Dioleylphosphite (2.91 g, 5 mmol) and histidine methylester dihydrochloride or histamine dihydrochloride (1.21 g, or 920.3 mg, 5 mmol) were dissolved in methanol (20 mL). Then $CBrCl_3$ (550 µL, 5.5 mmol) was added at 0°C under a nitrogen atmosphere followed by a slow addition of DIPEA (2.6 mL, 15 mmol). The solution was stirred for 2h at 0°C and one night at 20°C. After purification by silica column chromatography ($CHCl_3$/MeOH 100:0 to 80/20) these compounds were obtained as viscous oils. (yield: **1** = 29%, **2**= 30%).

O,O-dioleyl-N-(L-histidinemethylester)phosphoramidate 1

^{1}H NMR (500 MHz ; $CDCl_3$): 0.86 (t, 6H, CH_3, $^3J_{H-H}$ = 6.6Hz) ; 1.26 (m, 44H, CH_2) ; 1.65 (m, 4H, CH_2 β-O) ; 1.99 (m, 8H, CH_2 α-CH=CH) ; 3.08 (d, 2H, CH_2Im, $^3J_{H-H}$ = 5,3 Hz) ; 3.71 (s, 3H, OCH_3) ; 3.80 (m, NH) ; 3.98 (m, 4H, CH_2 α-O, $^3J_{H-H}$ = $^3J_{P-H}$ = 6.4Hz) ; 4.12 (m, CH(NH)) ; 5.32 (m, 4H, CH=CH) ; 6.80 (s, H) ; 7.53 (s, H) ; ^{13}C NMR (100 MHz ; $CDCl_3$): 14.1 (s, 2 CH_3) ; 22.6 to 32.0 (s, 28 CH_2) ; 30.3 (d, 2 CH_2 β-O, $^3J_{P-C}$ = 6.2 Hz) ; 30.9 (s, CH_2Im) ; 52,3 (s, OCH_3) ; 54.3 (s, CH(NH)) ; 66.1 (d, 2 CH_2 α-O, $^2J_{P-C}$ = 6.6 Hz) ; 115.4 (s, C) ; 129.6 (s, CH=CH) ; 129.7 (s, CH=CH) ; 131.5 (s, C) ; 134.8 (s, C) ; 172.8 (s, CO) ; ^{31}P NMR (121.49 MHz ; $CDCl_3$): 7.6 (s) ; ESI pour $C_{43}H_{81}N_3O_5P$, $[M + H]^+$, calcd 750.59139 found 750.5905.

O,O-dioleyl-N-(histamine)phosphoramidate 2

^{1}H NMR (500MHz ; $CDCl_3$): 0.86 (t, 6H, CH_3, $^3J_{H-H}$ = 6.6Hz) ; 1.26 (m, 44H, CH_2) ; 1.65 (m, 4H, CH_2 β-O) ; 1.99 (m, 8H, CH_2 α-CH=CH) ; 2.79 (t, 2H, CH_2Im, $^3J_{H-H}$ = 5.5 Hz) ; 3.13 (m, NH) 3.19 (m, 2H, CH_2(NH)) ; 3.98 (m, 4H, CH_2 α-O, $^3J_{H-H}$ = $^3J_{P-H}$ = 6.4Hz) ; 5.32 (m, 4H, CH=CH) ; 6.82 (s, H) ; 7.54 (s, H) ; ^{13}C NMR (100 MHz ; $CDCl_3$): 14.1 (s, 2 CH_3) ; 22.6 to 32.0 (s, 28 CH_2) ; 29.1 (s, CH_2Im) ; 30.3 (d, 2 CH_2 β-O, $^3J_{P-C}$ = 6.2 Hz) ; 41.4 (s, CH_2(NH)) ; 66.1 (d, 2 CH_2 α-O, $^2J_{P-C}$ = 6.6 Hz) ; 116.0 (s, C) ; 129.6 (s, CH=CH) ; 129.7 (s, CH=CH) ; 131.1 (s, C) ; 135.1 (s, C) ; ^{31}P NMR (121.49 MHz ; $CDCl_3$): 9.7 (s) ; ESI pour $C_{41}H_{79}N_3O_3P$, $[M + H]^+$, calcd 692.58591 found 692.5854.

Plasmids. pTG11033 (pCMV-Luc; 9514 bp) (kindly given by Transgene S.A., Strasbourg, France) was a plasmid DNA encoding the *firefly* luciferase gene under the control of the human cytomegalovirus promoter. Super coiled plasmid DNA was isolated by a standard alkaline lysis method, and purification was carried out with the QIAGEN Mega Kit (QIAGEN, Courtaboeuf, France).

Liposomes. A lipid film was prepared in a sterile round bottomed vessel (10 mL) under nitrogen by drying an ethanol mixture of one cationic lipid (10.8 mM, 0.5 mL) and one co-lipid (10.8 mM, 0.5 mL) (molar ratio of 1 : 1). The film was hydrated in sterile HEPES buffer (10 mM, 1 mL) at pH 7.4 vortexed for 3 min and kept at 4°C. After 2 h, the mixture was vortexed and sonicated for 15 min at 37 kHz using a Bioblock ultrasonic bath (Bioblock Scientific, Illkirch, France).

Lipoplexes. Liposomes (5.4 mM, 9 µL, 0.0486 µmol positive charge or 18 µL, 0.0972 µmol positive charge) were diluted in HEPES buffer (10 mM, 200 µL) at pH 7.4. After 15 min, pCMV-Luc (7.5 µg, 1.19×10^{-6} µmol, 0.0227 µmol negative charge) in HEPES buffer (10 mM, 20 µL) at pH 7.4 was added to the liposome solution, and the mixture was incubated for 30 min at room temperature. The lipoplex solution was completed to 1.5 mL with serum-free medium, and the NaCl concentration was adjusted to 0.15 M with a 5 M NaCl solution. When indicated, the lipoplex solution was completed to 1.5 mL with culture medium supplemented with 50% serum, and the NaCl concentration was adjusted to 0.15 M with a 5 M NaCl solution.

Measurements of Size and Zeta Potentials. Liposomes and lipoplexes were diluted to 1mL in 10mM HEPES buffer, pH 7.4. Their ζ potential was measured by electrophoretic mobility with ZetaSizer 3000 (Malvern Instruments, Orsay, France). The following parameters were set up: viscosity, 0.891 cP; dielectric constant, 79; temperature, 25°C; F(Ka), 1.50 (Smoluchowsky); maximum voltage of the current, 15 V. The system was calibrated with DTS 5050 standard from Malvern. Measurements were done ten times with the zero-field correction. The ζ potential was calculated with the Smoluchowsky approximation. The size of liposomes and lipoplexes was measured by quasi-elastic laser light scattering (QELS) with ZetaSizer 3000 in 10 mM HEPES buffer (pH 7.4) with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated with the 200 ± 5 nm polystyrene polymer (Duke Scientific Corps Palo Alto, CA). The diameter of liposomes and lipoplexes was calculated in the automatic mode.

Table S1: Size and ζ potential of liposomes

Size of Liposomes (nm)					
Cat-lipids	Co-lipids				
	-	DOPE	**1**	**2**	Chol
3	165 ± 21	169 ± 22	208 ± 12	246 ± 17	123 ± 25
4	143 ± 20	211 ± 3	240 ± 26	254 ± 40	72 ± 22
5	244 ± 56	269 ± 29	196 ± 15	126 ± 60	49 ± 8
DOTAP	35 ± 3	200 ± 25	44 ± 4 183 ± 30	58 ± 7 242 ± 70	140 ± 10
ζ of Liposomes (mV)					
Cat-lipids	Co-lipids				
	-	DOPE	**1**	**2**	Chol
3	76 ± 6	65 ± 2	59 ± 3	48 ± 2	44 ± 1
4	70 ± 2	53 ± 3	63 ± 7	42 ± 9	50 ± 2
5	69 ± 2	56 ± 1	33 ± 3	44 ± 7	46 ± 1
DOTAP	20 ± 4	38 ± 2	33 ± 1	42 ± 3	57 ± 1

Imidazolium phosphoramidate (**5**), Argininemethylester phosphoramidate (**3**), Homo-Argininemethylester phosphoramidate (**4**) or DOTAP liposomes were made without or either with cholesterol (chol), histamine phosphoramidate (**2**), histidinemethylester phosphoramidate (**1**) or DOPE as co-lipid. The cationic lipid to co-lipid molar ratio was 1/1.

Table S2: Size and ζ potential of lipoplexes

Size of Lipoplexes (nm)					
Cat-lipids	Co-lipids				
	-	DOPE	**1**	**2**	Chol
3	[b] 218 ± 29	[b] 203 ± 42	[b] 223 ± 15	[a] 300 ± 19	[a] 100 ± 48
4	[b] 239 ± 31	[b] 220 ± 23	[a] 237 ± 30	[a] 241 ± 18	[a] 233 ± 44
5	[b] 236± 20	[a] 231 ± 41	[b] 242 ± 12	[a] 280 ± 19	[b] 160 ± 30
DOTAP	[b] 77 ± 23 243 ± 20	[b] 125 ± 10 293 ± 10	ND	[b] 505 ± 55	[b] 160 ± 50
ζ of Lipoplexes (mV)					
Cat-lipids	Co-lipids				
	-	DOPE	**1**	**2**	Chol
3	[b] 64 ± 11	[b] 62 ± 6	[b] -6.5 ± 3	[a] 3 ± 6	[a] -41 ± 2
4	[b] 75 ± 2	[b] 50± 3	[a] -1 ± 2	[a] -0.5 ± 2	[a] 31 ± 1
5	[b] -1.2 ±0.2	[a] -10 ± 4	[b] 26 ± 2	[a] -14 ± 1	[b] 43 ± 1
DOTAP	[b] 31 ± 1	[b] 37 ± 1	[b] 11 ± 1	[b] 12 ± 1	[b] 35 ± 2

Imidazolium phosphoramidate (**5**), Argininemethylester phosphoramidate (**3**), Homo-Argininemethylester phosphoramidate (**4**) or DOTAP liposomes were made without or either with cholesterol (chol), histamine phosphoramidate (**2**), histidinemethylester phosphoramidate (**1**) or DOPE as co-lipid. The cationic lipid to co-lipid molar ratio was 1/1. The lipid/DNA charge ratios corresponding to lipoplexes that gave the best transfection efficiencies were [a]2 or [b]4.

Cells and Cell Culture. Human Embryo Kidney HEK293-T7 cells (kindly given by Drs. L. Huang and M. Brisson, University of Pittsburgh, Pittsburgh, PA)[4] were cultured in DMEM supplemented with Fetal Bovine Serum (FBS) (10%), L-glutamine (2 mM) (Life Technologies), sodium pyruvate (1 mM) (Life Technologies), penicillin (100 units/mL) (Life Technologies) and streptomycin (100 Units/mL) (Life Technologies) and geneticin (400 μg/mL. Cells were mycoplasma free, as evidenced by the bis-benzimidazole (Hoechst 33258, Molecular Probes) method.[5]

Transfections. Two days prior to transfection, cells were seeded at 1 x 10^5 cells per 2 cm^2 in culture medium (1 mL) in a 24-well plate. At the time of the experiment, cell cultures were 80% confluent. Cells were washed two times with serum-free culture medium before incubation with lipoplexes. Then, the lipoplex solution (0.5 mL, 2.5 μg pDNA) was added in each well and cells were incubated at 37 °C. After 4 h, the medium was removed and cells were cultured 48 h at 37 °C in complete culture medium.

Luciferase Assay. For measuring luciferase gene expression, the luminescence activity was monitored according to De Wet et al.[6] The medium was discarded, and cells were washed three times with PBS. The homogenization buffer (200 μL) that contained Tris-phosphate (25 mM, pH 7.8), $MgCl_2$ (8 mM), DTT (1 mM), EDTA (1 mM), Triton X-100 (1%), and glycerol (15%) was poured into each well, and tissue culture plates were kept for 15 min at 20 °C. The solution was recovered and spun down (5 min at 800 g). ATP (2 mM) in the homogenization buffer without Triton X-100 (95 μL) was added to supernatant (60 μL), and the solution was shaken with a vortex. The luminescence was recorded for 4 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany) upon addition of luciferin (167 mM) in water (150 μL). Measurements were done in duplicate. The number of RLU of 1 pg/mL of luciferase was 2000 under these conditions. The data shown correspond to the number of relative light units (RLU) per mg proteins. Proteins were determined on each sample by a modified bicinchoninic acid (BCA) colorimetric assay.[7,8] Statistical comparisons were performed by Student's *t* test.

[4] Brisson, M. ; Tseng, W.C. ; Almonte, C. ; Watkins, S. ; Huang, L. *Hum. Gene Ther.* **1999**, *10*, 2601-2613.

[5]Chen, T.R. *Exp Cell Res* **1977**, *104*, 255-262.

[6]de Wet, J.R. ; Wood, K.V. ; DeLuca, M. ; Helinski, D.R. ; Subramani, S. *Mol. Cell. Biol*. **1987**, *7*, 725-737.

[7]Smith, P.K. ; Krohn, R.I. ; Hermanson, G.T. ; Mallia, A.K. ; Gartner, F.H. ; Provenzano, M.D. ; Fujimoto, E.K. ; Goeke, N.M. ; Olson, B.J. ; Klenk, D.C. *Anal. Biochem.* **1985**, *150*, 76-85.

[8]Hill, H.D. ; J. G. Straka,J.G. *Anal. Biochem.* **1988**, *170*, 203-208.

Measurement of EGFP positive cells:

Forty eight hours upon transfection with pCMV-EGFP lipoplexes, cells were harvested by treatment with trypsin, washed in complete medium and the pellet was suspended in sheath fluid. Then, the cell-associated fluorescence intensity was measured by flow cytometry (FACSort, Becton Dickinson) (λexc = 488 nm; λem = 520 nm).

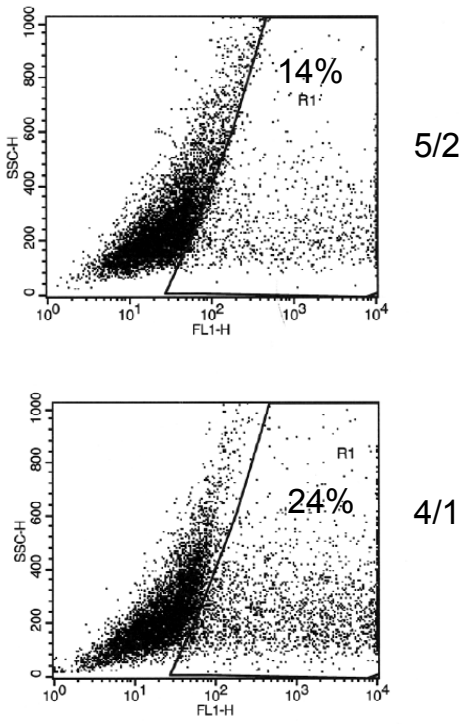

Figure S1: Assessment of the number of transfected HEK293-T7 cells. Cells were transfected with **5/2** and **4/1** liposomes complexed with pCMV-EGFP (2.5 μg). Upon 48 h of culture, the number of cells expressing EGFP was measured by flow cytometry (λexc = 488 nm; λem = 520 nm). The lipid/DNA charge ratio was 2.

Toxicity Assay: The cell viability was evaluated with the colorometric 3-[4,5]-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.[9] Forty eight hours upon transfection, MTT (5 mg/ml in PBS, 50 μL) was added to cell culture and incubated for 4 h at 37 °C. MTT converted to an insoluble dye in living cells was then solubilised with acidic isopropanol. The absorbance was measured at 570 nm. The percentage of cytotoxicity was $(A_0 - A)/A_0$ x 100 where A was the absorbance of transfected cells and A_0 was the absorbance measured for untransfected cells cultured under the same conditions as those used for transfected cells.

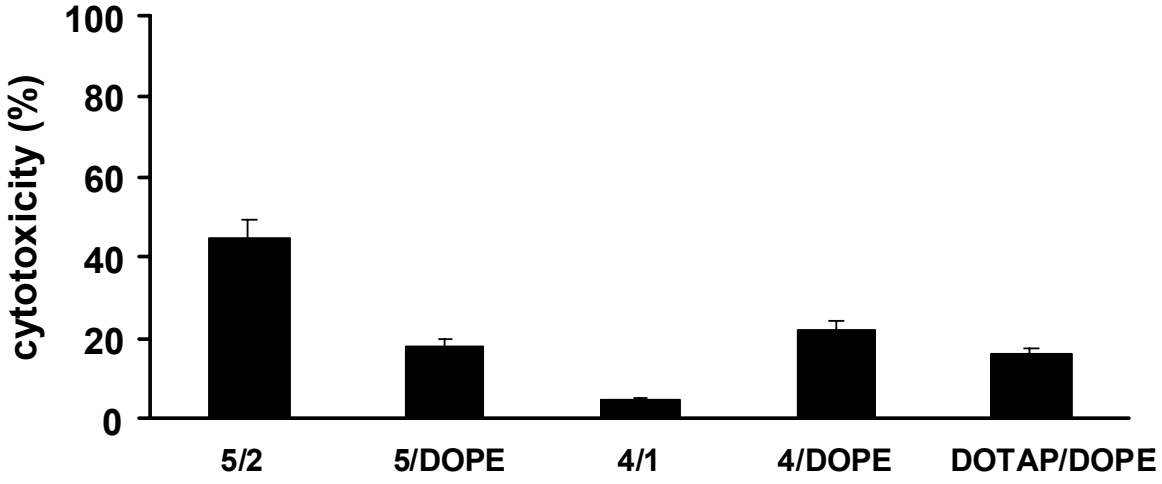

Figure S2: Cytotoxicity of lipoplexes. Colorimetric MTT assay-based percent cell viabilities after transfection with the various lipoplexes. The lipid/DNA charge ratio was 2 for **5/2, 5/DOPE and 4/1** and 4 for the other lipoplexes.

[9] Mosmann, T. *J. Immunol. Methods* **1983** . *65*, 55-63.

FRET experiments. Intermixing of membrane lipids was measured with the fluorescence resonance energy transfer (RET) assay. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (NBD-PE) (Molecular Probes, Eugene, OR) and *N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (Rho-PE) (Molecular Probes) were used as donor and acceptor fluorescent lipids, respectively. PC/PS/NBD-PE/Rho-PE (molar ratio: 50:40:5:5) liposomes et 5.4 mM were prepared as followed. 1,2 dimiristoyl-*sn*-glycero-3-phosphocholine (PC) (2.68 mmole) plus 1,2 dimiristoyl-*sn*-glycero-3-[phospho-L-serine] (PS) (2.16 mmole) plus NBD-PE (0.27 mole) plus Rho-PE (0.27 mmole) dissolved in chloroform/methanol was dried under reduced pressure. The dried lipid film was hydrated for overnight at 4°C in 1 ml of 10 mM Hepes buffer, pH 7.4. The suspension was vigorously vortexed for 2-5 min at room temperature and sonicated for 15 min in cold bath sonicator at 35 KHz (Bioblock Scientific, Strasbourg, France). Fusion was induced by adding aliquots of 5.4 mM liposomes (**5** with either **2** or DOPE) or (**4** with either **1** or DOPE) to the PC/PS/NBD-PE/Rho-PE liposomes suspension (20 μM) in 10 mM Hepes buffer at pH 7.4. or pH 6.0 directly in the cuvette. The fluorescence intensity of Rho-PE was monitored for 10 min with a spectrofluorometer (Shimadzu 5000). The excitation was at 465 nm and emissions were at 530 nm and 580 nm for NDB and Rho, respectively. All experiments were done at 25°C. Control was performed with PC/PS (molar ratio: 55:45) liposomes. 100% fusion was obtained by adding triton X-100 (30 μl of a 1% solution).

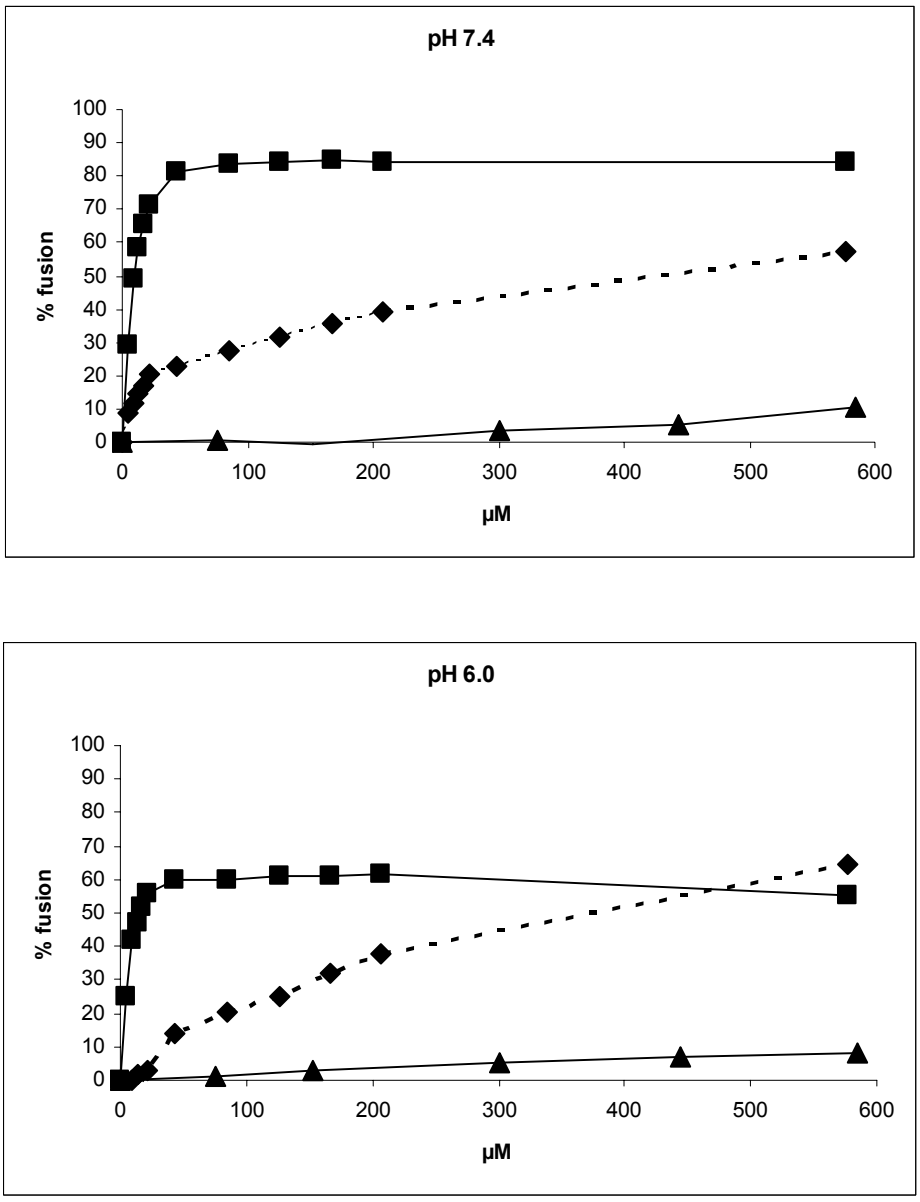

Figure S3: Fusion of liposomes with membrane models at pH 6.0 and pH 7.4. :◆ **4** with DOPE 1:1 ; ■ : **4** with **1** 1:1 ; ▲: PC/PS