Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supplementary Information

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Materials and equipments. Protected amino acids were obtained from Activotec (Cambrigde, UK), Bachem Biochimie SARL (Voisins-les-Bretonneux, France), France Biochem SA (Meudon, France), Merck Eurolab (Fontenay-sous-Bois, France) or Calbiochem-Novabiochem (Merck Biosciences - VWR, Limonest, France). PyBOP® was purchased from Calbiochem-Novabiochem and PyAOP® from PerSeptive Biosystems (Foster City, Canada). NovaSyn® TGR resin were obtained from Calbiochem-Novabiochem, Fmoc-Gly-SASRIN® resin from Bachem Biochimie SARL and 2-chlorotritylchloride® resins from Advanced ChemTech Europe. Other reagents were obtained from Aldrich (Saint-Quentin Fallavier, France) and Acros (Noisy-le-Grand, France).

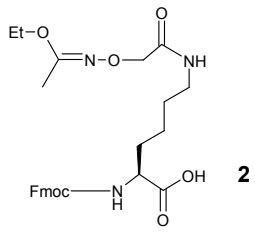
RP-HPLC analyses were performed on Waters equipment consisting of a Waters 600 controller, a Waters 2487 Dual Absorbance Detector and a Waters In-Line Degasser. The analytical column used was the Nucleosil 120 Å 3 μm C18 particles, 30 × 4 mm^2 operated at 1.3 $mL.min^{-1}$ with linear gradient programs in 15 min run time (classical program 5 to 100 % B in 15 min). UV monitoring was performed most of the time at 214 nm and 250 nm. Solvent A consisted of H_2O containing 0.1% TFA and solvent B of CH_3CN containing 9.9% H_2O and 0.1% TFA. Water was of Milli-Q quality and was obtained after filtration of distilled water through a Milli-Q® cartridge system. CH_3CN and TFA were of HPLC use quality. RP-HPLC purifications were performed on Waters equipment consisting of a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. The preparative column, Delta-Pak™ 300 Å 15 μm C18 particles, 200 × 25 mm^2 was operated at 22 $mL.min^{-1}$ with linear gradient programs in 30 min run time. Solvents A and B were the same than the ones used in RP-HPLC analysis.

Electron spray ionization (ESI-MS) mass spectra were obtained on an Esquire 3000 (Bruker). NMR spectra were recorded on BRUKER Avance 300 spectrometers. Chemical shifts are expressed in ppm and calculated taking the solvent peak as an internal reference.

General procedures for peptide syntheses. Assembly of all linear protected peptides was performed manually or automatically by solid-phase peptide synthesis (SPPS) using the standard 9-fluorenylmethoxycarbonyl/tertiobutyl (Fmoc/*t*Bu) protection strategy. In manual SPPS, device consisted in a glass reaction vessel fitted with a sintered glass frit. The latter allowed elimination of excess reagents and solvents under compressed air. Before use, the vessel was treated for 12 h (typically overnight) with $(CH_3)_2SiCl_2$ as lubricant to prevent resin beads from sticking to the glass inner wall during the synthesis. It was then carefully washed with CH_2Cl_2 until complete acid trace clearance. At the beginning of the synthesis and after each ether washing, the resin was washed and swollen twice with CH_2Cl_2 (20 mL/g resin) for 15 min and once with DMF (20 mL/g resin) for 15 min. Coupling reactions were performed using, relative to the resin loading, 1.5-2 eq. of N_α-Fmoc-protected amino acid *in situ* activated with 1.5-2 eq. PyBOP and 3-4 eq. DIPEA in DMF (10 mL/g resin) for 30 min. The resin was then washed twice with DMF (20 mL/g resin) for 1 min and twice with CH_2Cl_2 (20 mL/g resin) for 1 min. The completeness of amino acid coupling reaction was checked by two tests: Kaiser and TNBS. *N*α-Fmoc protecting groups were removed by treatment with piperidine/DMF (1:4) (10 mL/g resin) for 10 min. The process was repeated three times and the resin was further washed five times with DMF (10 mL/g resin) for 1 min. The completeness of the deprotection was checked by UV measurement (λ = 299 nm, ε = 7800 $M^{-1}.cm^{-1}$).

Synthesis of lipopeptide 1

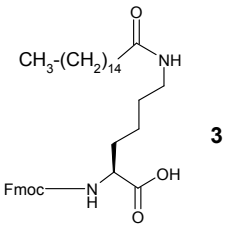
Building block 2.



2

Amino acid **2** was prepared following the procedure previously described.[1]

Building block 3.



3

To a stirred solution of palmitic acid (1 g, 3.9 mmol) and *N*-hydroxysuccinimide (0.6 g, 5.2 mmol) in ethyl acetate/dioxane (12 mL, 1:1) at 4 °C was added DCC (0.8 g, 3.9 mmol) in one portion. The resulting mixture was stirred at room temperature for 3 h. The formed DCU was filtered off and the filtrate concentrated under vacuum. The obtained residue was dissolved in CH_2Cl_2 (80 mL), and the solution was washed with 5% aqueous $NaHCO_3$ (75 mL), and water (3 x 75 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated. NHS ester of palmitic acid was obtained as white powder which was used without further purification (1.2 g, 3.4 mmol, 87 %). ^{1}H NMR (300 MHz, $CDCl_3$): □ 0.90 (3H, t, J = 6.7 Hz), 1.28 (22H, m), 1.77 (2H, m), 1.45 (2H, t, J = 7.5 Hz), 2.85 (4H, m).

To a stirred mixture of Fmoc-lysine (0.82 g, 1.7 mmol) and DIPEA (0.3 mL, 1.7 mmol) in CH_2Cl_2 (14 mL) at room temperature was added dropwise over 10 min a solution of NHS ester (0.6 g, 1.7 mmol) in CH_2Cl_2 (6 mL). The pH of the resulting mixture was regularly adjusted to pH 8-9 by

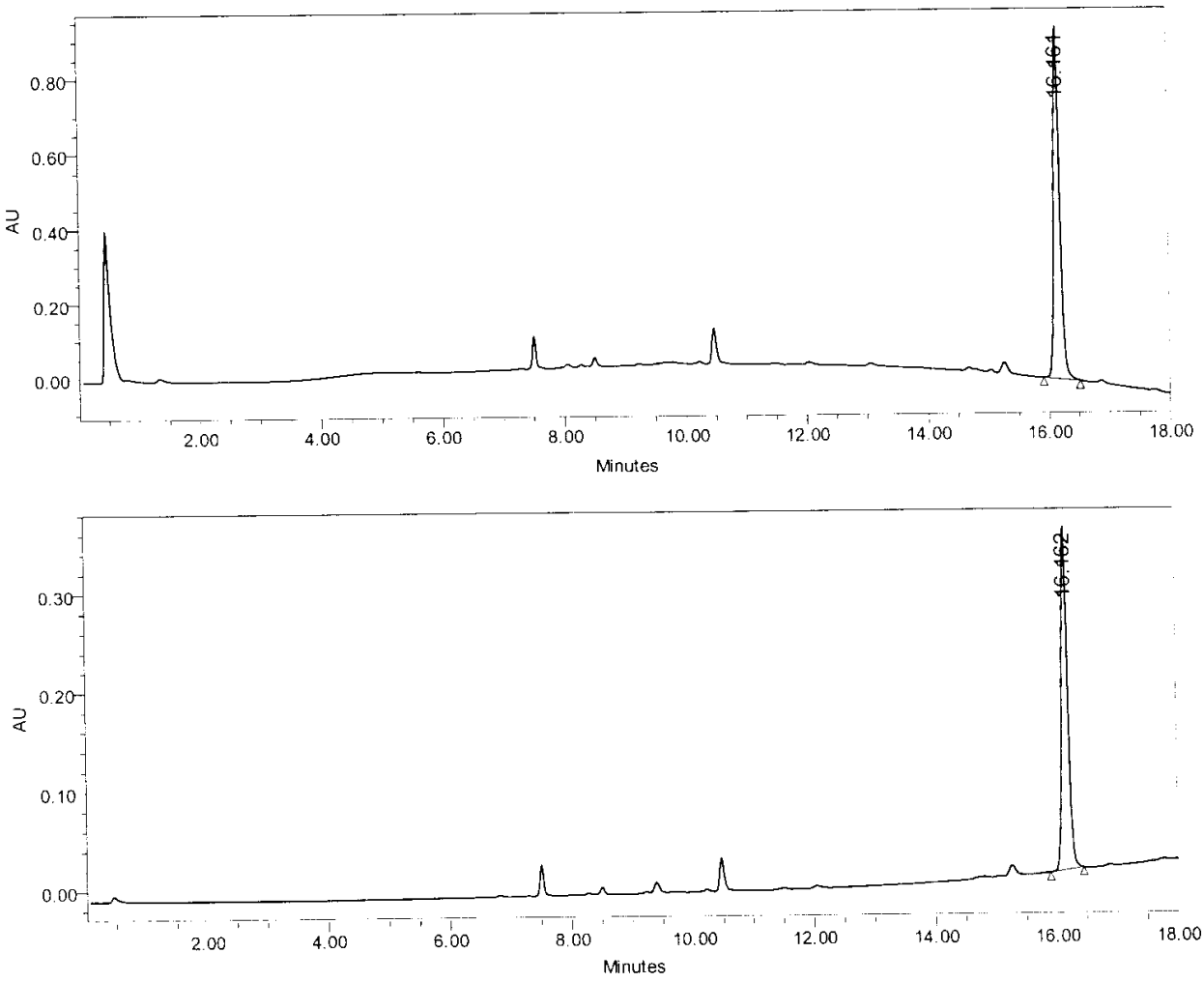
1 S. Foillard, M. Ohsten Rasmussen, J. Razkin, D. Boturyn and P. Dumy, *J. Org. Chem.*, 2008, **73**, 983.

further additions of DIPEA. After 1 h of reaction, the reaction mixture was concentrated under vacuum providing an oily residue. After addition of CH_2Cl_2 (15 mL), the organic phase was washed with hydrochloric acid solution (1 M, 2 x 12 mL), water (2 x 12 mL), and brine (12 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated. Lyophilization from water/CH_3CN afforded the building block **3** as a white solid which was used without further purification (0.82 g, 1.4 mmol, 82 %). ^{1}H NMR (300 MHz, $CDCl_3$): □ 0.80 (3H, t, J = 6.7 Hz), 1.15-1.18 (22H, m), 1.33-1.56 (10H, m), 2.06 (2H, t, J = 7.7 Hz), 3.16 (2H, m), 3.60 (1H, m), 4.14 (1H, t, J = 7.4 Hz), 4.28 (2H, m), 5.70 (1H, d, J = 6.8 Hz), 7.23 (2H, m), 7.30 (2H, m), 7.53 (2H, m), 7.68 (2H, m).

RP-HPLC profile of 3

(Nucleosil 120 Å 3 µm C18 particles, 30 × 4 mm^2)

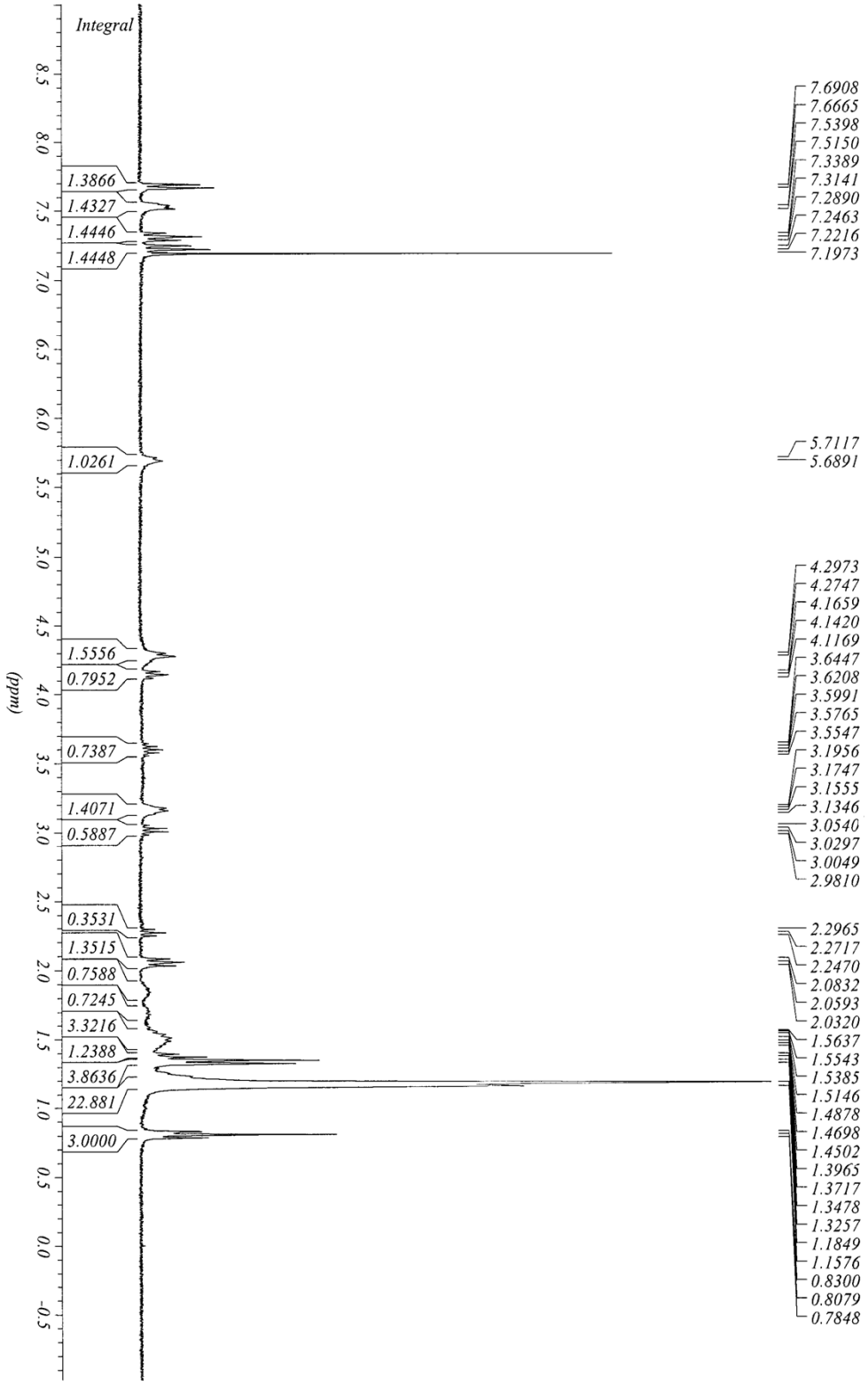
Conditions: see Materials and equipments pS2 214 nm and 250 nm



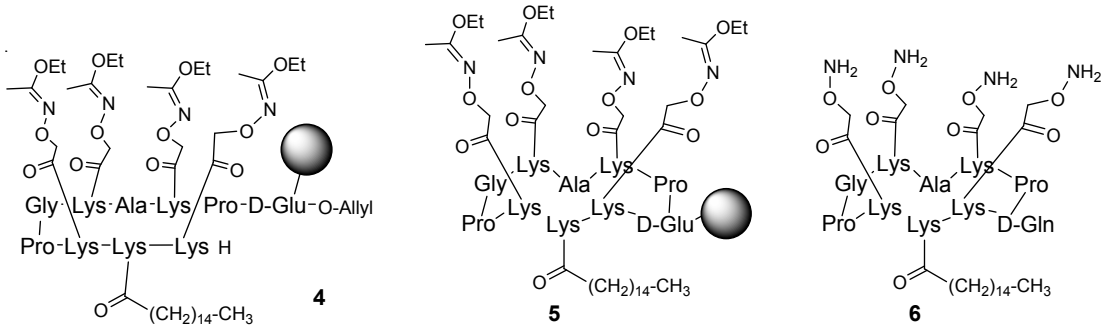
1H-RMN of 3

($CDCl_3$)

Conditions: see Materials and equipments pS2



Lipodecapeptide 5.

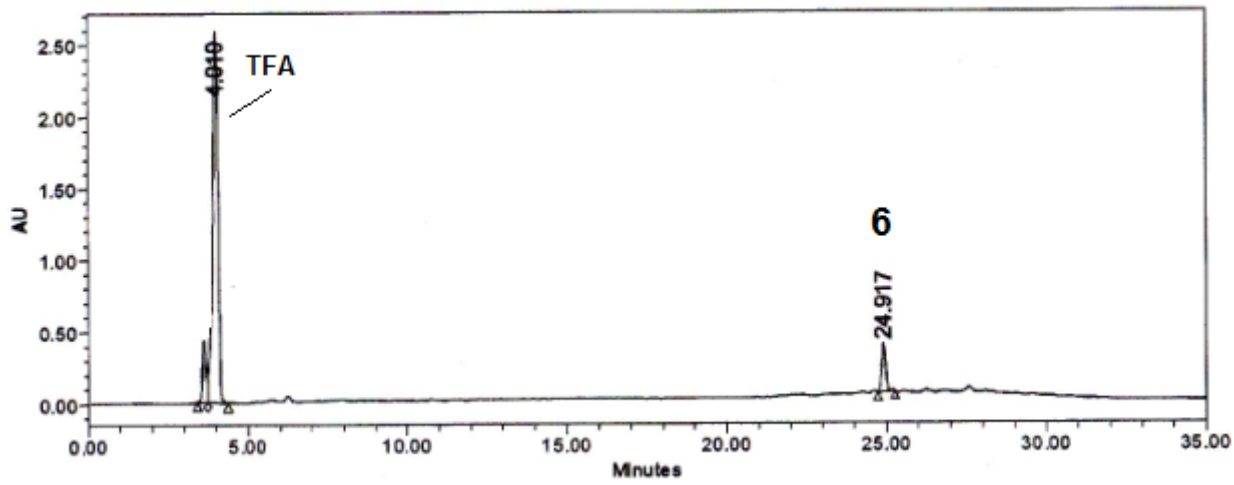


Lipopeptide **4** synthesis was carried out on Rink-amide resin (150 mg, loading 0.3 mmol/g) as described in the general procedure. Then, allyl group was removed: the linear decapeptide **4** on resin (150 mg, 0.037 mmol), previously swelled in dry CH_2Cl_2 under argon (10 mL, 15 min), was treated by adding successively phenylsilane (4.5 mL, 3.7 mmol) followed after 3 min by $Pd(PPh_3)_4$ (15 mg, 0.013 mmol). The reaction mixtures were stirred for 30 min at room temperature. The resin was washed with CH_2Cl_2 (2 x 10 mL), dioxane/H_2O (9/1, 10 mL), DMF (10 mL) and CH_2Cl_2 (10 mL). Peptide cyclization was performed in DMF (8 mL). pH was adjusted to 8-9 by addition of DIPEA, then PyAOP (77 mg, 0.074 mmol) was added and the mixture was stirred at room temperature for 30 min. The resin was washed with DMF (5 x 10 mL) and CH_2Cl_2 (10 mL). Then, resin was treated with a solution of trifluoroacetic acid/H_2O/triisopropylsilane (90/5/5, 10 mL) for 30 min. Solvent was removed under reduced pressure and residue dissolved in the minimum of CH_2Cl_2. Ether was added to precipitate the crude lipopeptide **6**. The latter was triturated and washed three times with ether affording lipopeptide **6** as a white powder (30 mg, 0.014 mmol, 39%). ESI-MS calc for $C_{74}H_{132}N_{20}O_{20}$ 1620.99, found 1620.5.

RP-HPLC profile of 6

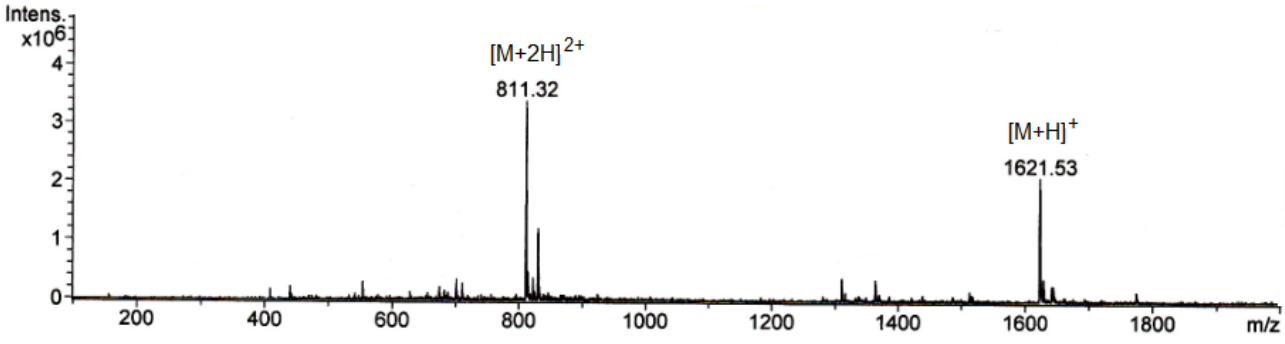
(Supelco 300 Å 10 µm C5 particles, 250 × 4.6 mm^2)

Conditions: linear gradient 5 to 100 % B in 30 min, 214 nm

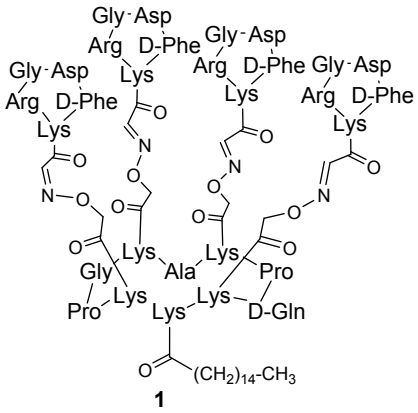


ESI-MS analysis of compound 6

Conditions: see Materials and equipments pS2



Palmitoyl-RAFT-RGD$_4$ (1).



1

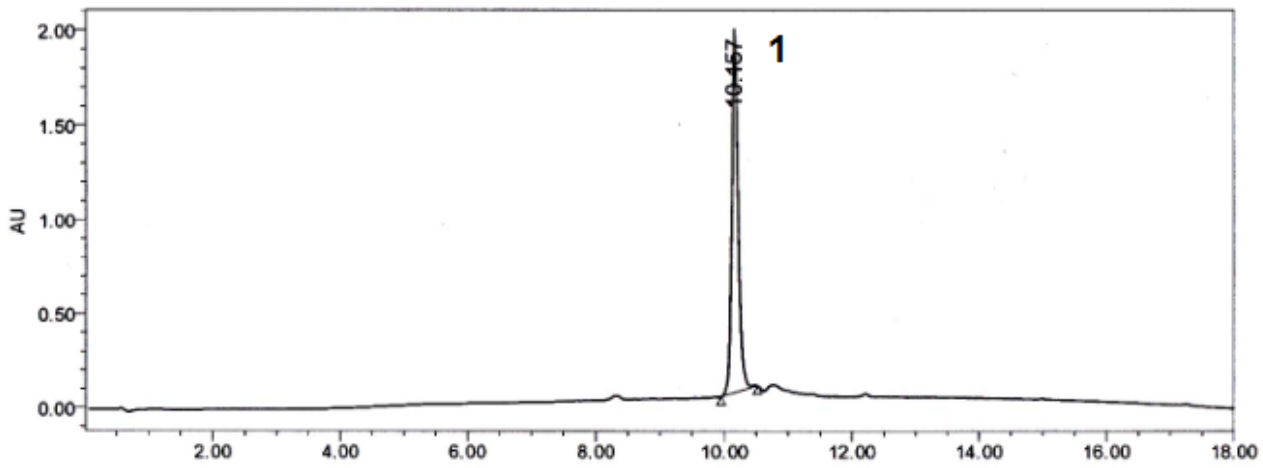
Cyclo[-Arg-Gly-Asp-D Phe-Lys(-CO-CHO)-] was prepared as previously described.[2] To a stirred solution of lipopeptide **6** (7.8 mg, 3.8 μmol) in acetic acid/H_2O/CH_3CN (50/25/25, 0.5 mL) was added Cyclo[-Arg-Gly-Asp-D Phe-Lys(-CO-CHO)-] (13.2 mg, 16.7 μmol). After 5 h stirring at room temperature, RP-HPLC purification afforded the compound **1** as a white powder (6.5 mg, 1.39 μmol, 37 %). ESI-MS calc for $C_{190}H_{288}N_{56}O_{52}$ 4188.17, found 4187.8.

2 D. Boturyn and P. Dumy, *Tetrahedron Lett.*, 2001, **42**, 2787.

RP-HPLC profile of Palmitoyl-RAFT-RGD$_4$ (1)

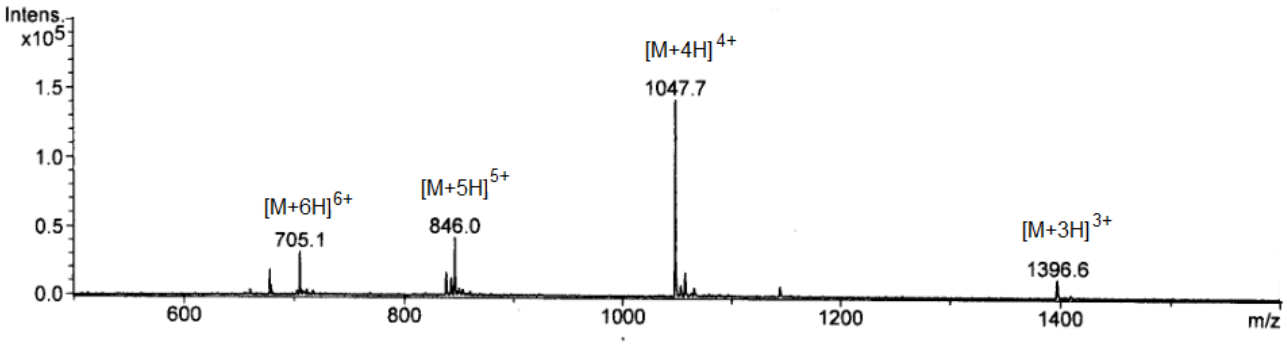
(Nucleosil 120 Å 3 µm C18 particles, 30 × 4 mm^2)

Conditions: linear gradient 5 to 100 % B in 15 min, 214 nm

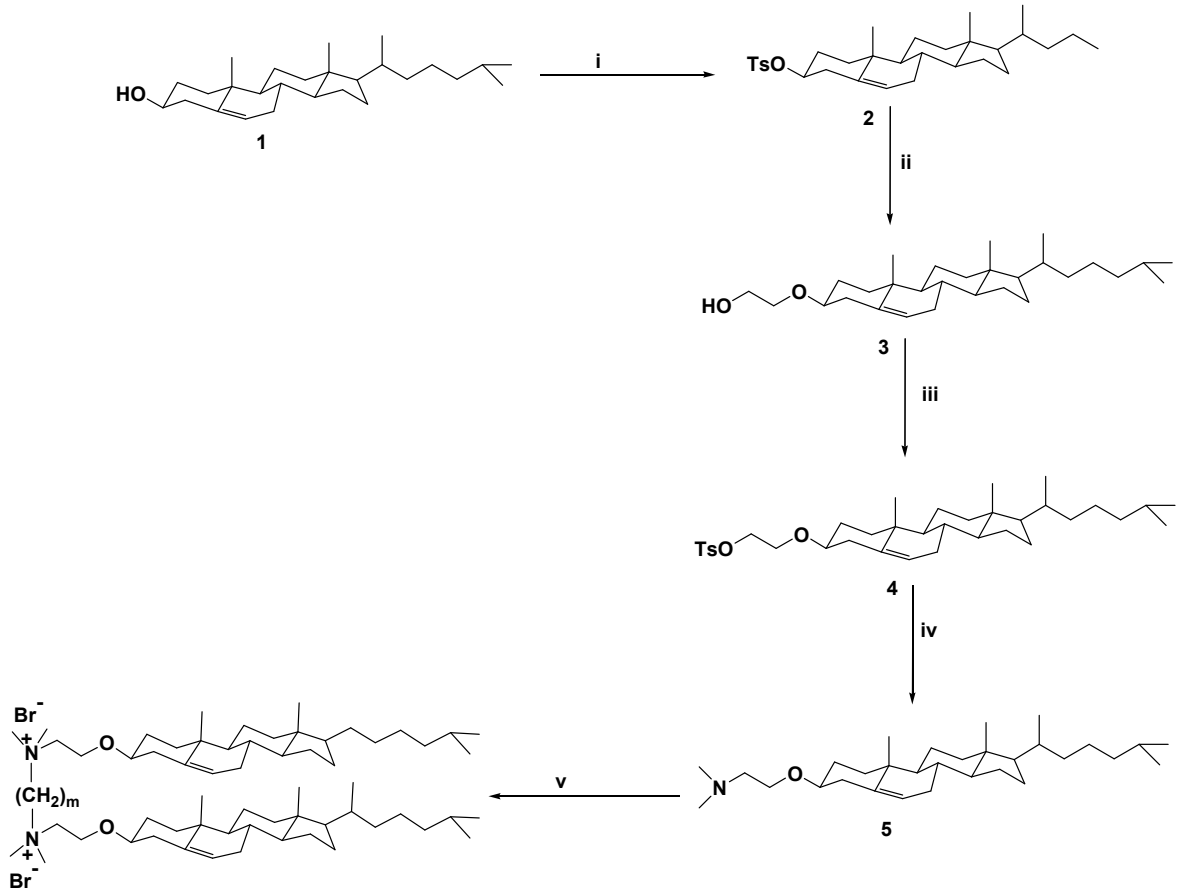


ESI-MS analysis of compound Palmitoyl-RAFT-RGD$_4$ (1)

Conditions: see Materials and equipments pS2



Synthesis of gemini lipids (GLs).



m = 5 (GL5)

Reagents, conditions and yields: (i) *p*-TsCl/Py/$CHCl_3$, 0 ^oC, 6 h, 92%; (ii) ethylene glycol, 1,4-dioxane, 4 h, reflux, 85%; (iii) *p*-TsCl/Py/$CHCl_3$, 0 ^oC, 6 h, 90%; (iv) Dimethyl amine, MeOH, 80 ^oC, 24 h, screw-top pressure tube, quantitative; (v) MeOH-EtOAc, *1,5*-dibromo alkanes, 80 ^oC, screw-top pressure tube, 48-72 h, 50-60%.

Characterization of gemini lipid (GL). The new gemini lipid was fully characterized by ^{1}H NMR, mass spectrometry and C, H, N analysis. Pertinent spectroscopic and analytical data are given below.

Lipid GL5: ^{1}H NMR ($CDCl_3$, 300 MHz): δ: 0.67 (*s*, 6H, 2 x C*H*$_3$, from cholesterol), 0.85-2.36 (*m*, 88H, cholesterol protons and -N^+-CH_2-(C*H*$_2$)$_3$-CH_2-N^+-), 3.2 (*m*, 2H, 2 x -C*H*-O-), 3.37 (br *s*, 12H, 2 x -N^+-(C*H*$_3$)$_2$), 3.80 (br. *s*, 4H, 2 x -O-C*H*$_2$-) 3.90 (br. *s*, 8H, 4 x -C*H*$_2$-N^+-), 5.35 (*d,* 2H, 2 x -C*H*=C-, *J* = 4.5Hz). ESI-MS: 492.4 (M^{+2}/2), 1064.7, 1066.9 (M^{+2} + Br^-). Elemental analysis (%) for $C_{67}H_{122}N_2O_2Br_2$: calcd: C 70.13, H 10.72, N 2.44; found C 70.24, H 10.52, N 2.83.

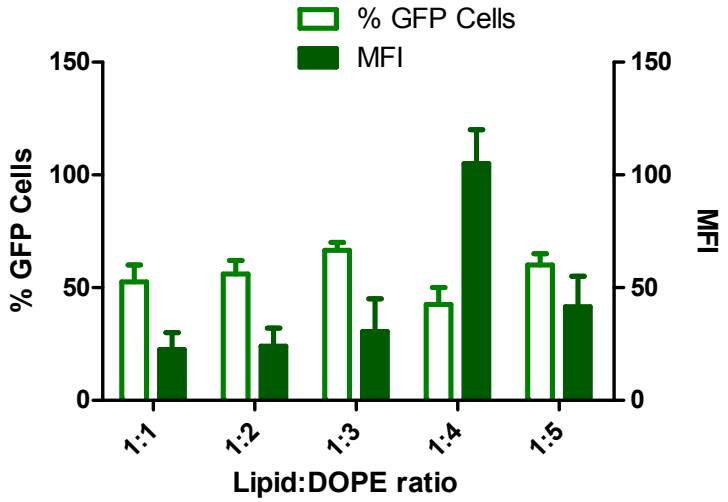


Figure S1: Transfection efficiencies of gemini lipid (GL5) with various mole ratios of DOPE using pEGFP-C3 plasmid DNA. Concentration of the DNA = 0.8 μg/well and lipid were used at N/P ratio of 0.5. Data are expressed as % GFP cells and MFI as obtained from flow cytometry analysis.

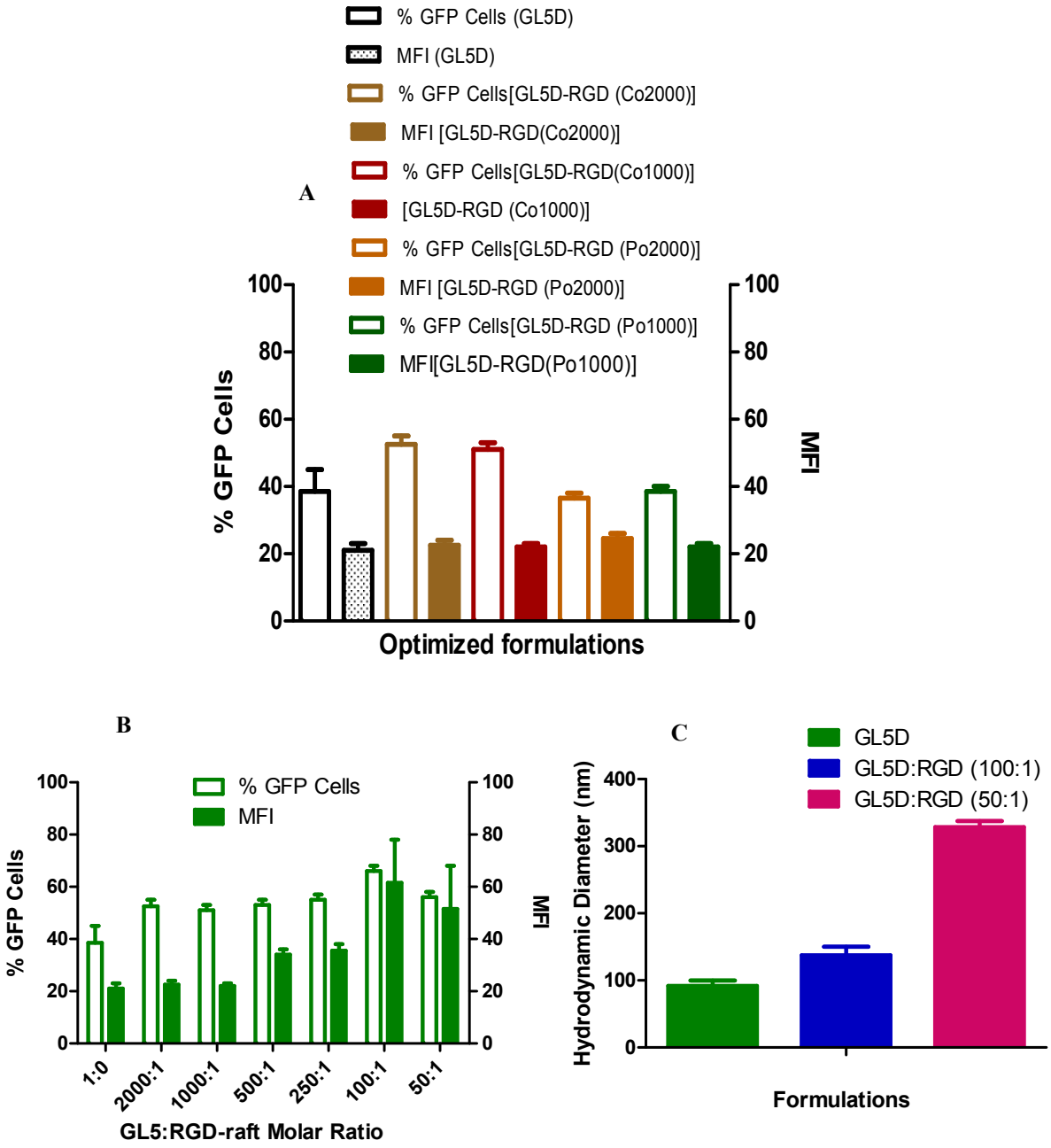


Figure S2. FACS data show an optimization of (A) the methodology used for a non-covalent inclusion of the RGD-RAFT in GL5D; (B) the molar ratio of GL5D and RGD-RAFT in co-liposomal formulations (C) significant changes in the hydrodynamic diameter of GL5D on incorporation of different molar concentrations of RGD-raft in GL5D formulations. Experiments were performed using 0.8 μg of DNA at N/P charge ratio 0.5 in presence of 10% FBS (-FBS+FBS) on HeLa cells.

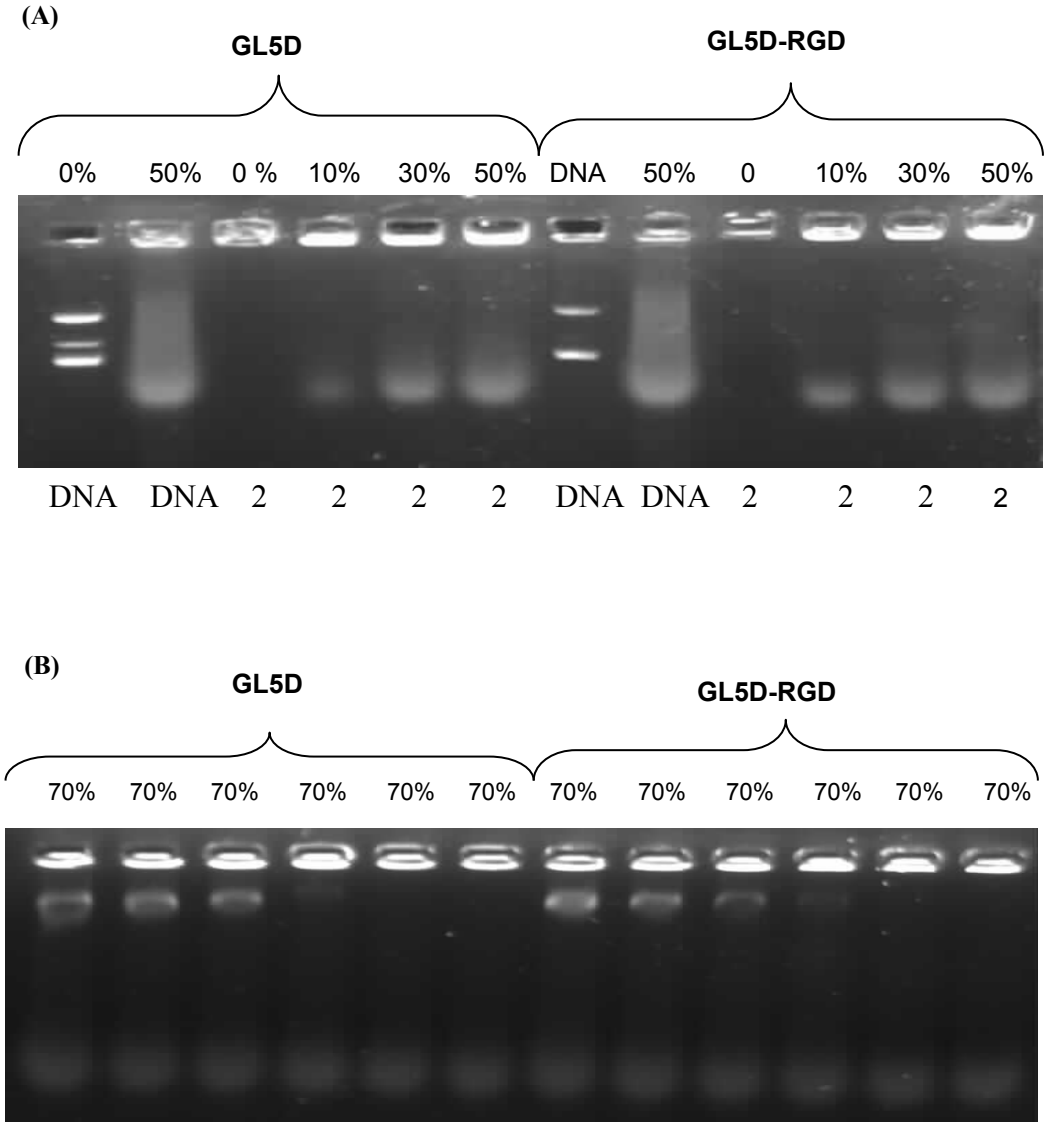


Figure S3. Stability of lipoplexes in presence of fetal bovine serum (FBS). Values above the gel shows percentage concentration of FBS while values below the gel shows N/P charge ratio.

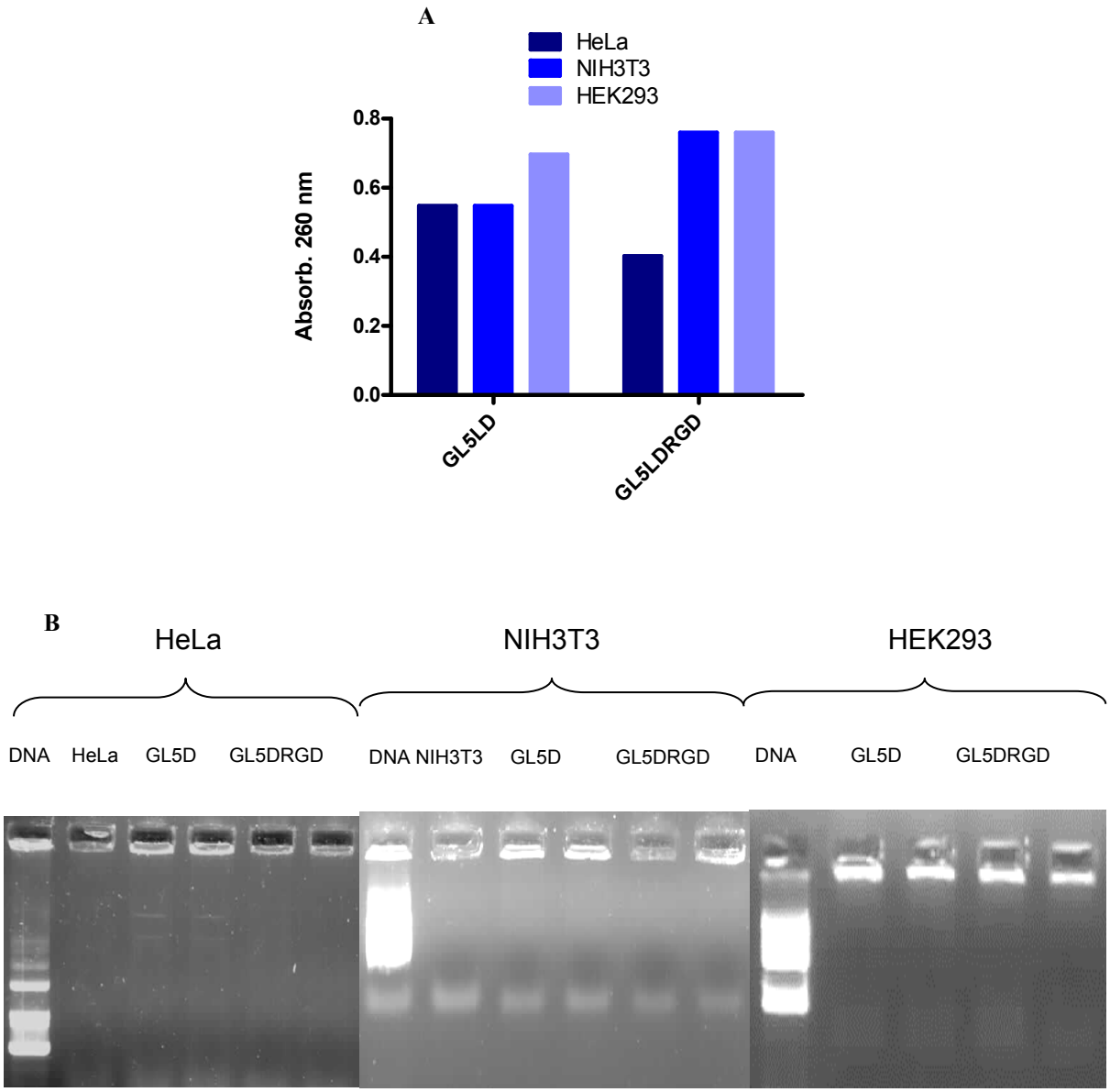


Figure S4. UV absorbance (Abs. 260 nm) of DNA obtained from lipoplexes remained outside the cells after 6 h of incubation with cells during transfection. Experiment was performed by using 0.8 μg of DNA and lipid/DNA charge ratio of 0.5.

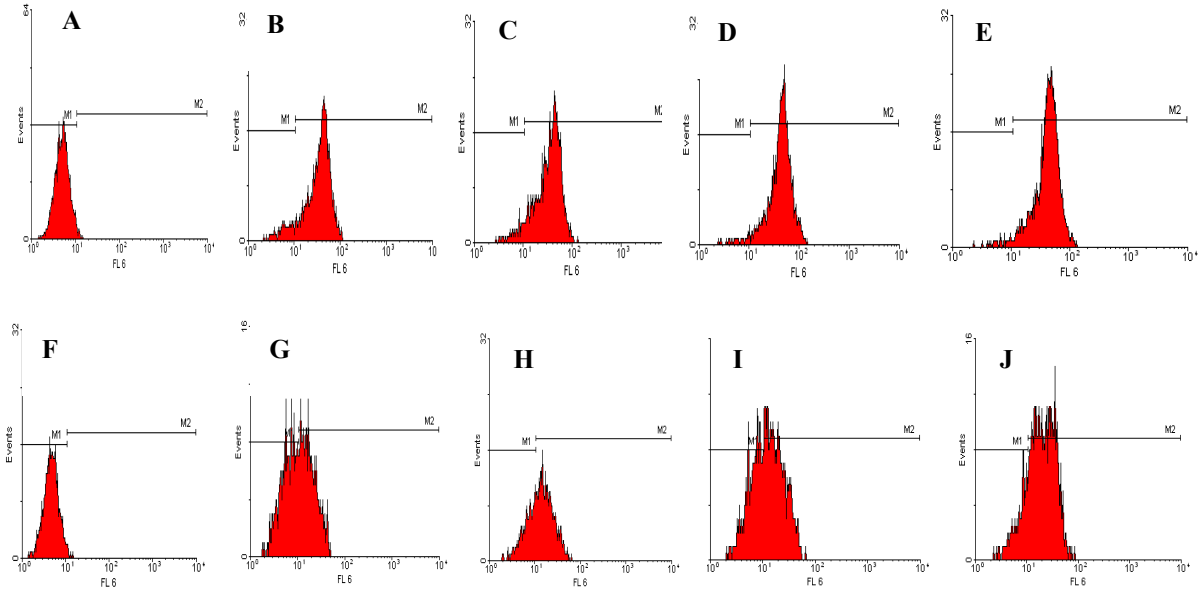


Figure S5. Surface binding efficiency of GLD-RGD-pyrene in HeLa (A-E) and NIH3T3 (F-J) cells. Concentration of GLD in GLD-RGD-pyrene added to the cells was (A) 0; (B) 1.5 μM; (C) 3.0 μM; (D) 4.5 μM; (E) 6.0 μM; (F) 0; (G) 1.5 μM; (H) 3.0 μM; (I) 4.5 μM; (J) 6.0 μM. Experiment was performed on 24h grown HeLa and NIH3T3 cells where cells were incubated with formulations for 2h.