Electronic Supplementary Material (ESI) for Biomaterials Science.


# Understanding and optimising the transfection of lipopolyplexes formulated in saline: the effects of peptide and serum

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**Supporting Information**

**Peptide synthesis**

Fmoc-Gly-NovaSyn TGT resin (0.21 mmol·g$^{-1}$, 150 mg, 31.5 µmol) was purchased from Merck Chemicals Ltd (Nottingham, UK). Novabiochem® Fmoc-protected amino acids were obtained from Merck Millipore (Darmstadt, Germany). Peptide coupling reagent O-benzotriazole-*N*,*N*,*N*',*N*'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and reaction catalyst, *N*,*N*-diisopropylethylamine (DIPEA) were supplied by Sigma-Aldrich (Poole, UK) as where piperidine, HPLC-grade dimethylformamide (DMF), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), 1,2-ethanedithiol (EDT), water (HPLC- grade) and diethyl ether.

**2.2.1 Peptide Synthesis**

All peptides were synthesised by Standard Fmoc Solid Phase Peptide Synthesis (SPPS) methodology. SPPS synthesis was performed in reaction syringes housed in a MultiSynTech Syro I automated system (Witten, Germany). Fmoc-Gly-NovaSyn TGT resin (0.21 mmol·g$^{-1}$, 150 mg, 31.5 µmol) was loaded into the reaction syringes and the resin pre-swelled in 3 mL DMF (HPLC-grade) for at least 30 min prior to the start of the synthesis. The total volume of all reagents in the reaction syringe was maintained as 1.5 mL. All reagents used for the peptide synthesis were dissolved in DMF (HPLC-grade). The MultiSynTech Syro I automated system was programmed with the following automatic deprotection/coupling cycle:

**Cycle one: deprotection**

The resin-loaded reaction syringe was filled with 1.5 mL of 40% v/v piperidine in DMF. The mixture contained in the reaction syringe was then automatically agitated for 20 s every minute for a total of 3 min after which the reagent was removed by filtration under vacuum and the resin was washed with DMF (4 × 1.5 mL). 0.75 mL of 40% v/v piperidine in DMF was then added to the reaction syringe followed by a further 0.75 mL of DMF to make a 20% v/v solution of piperidine in DMF. The mixture in the reaction syringe was agitated for 20 s every minute for a total of 10 min after which time the reagents were removed by filtration under vacuum and the resin was washed with 6 aliquots of 1.5 mL DMF.

**Cycle two: coupling**

The reaction syringe was next filled with the required Fmoc-protected amino acid (0.600 mL, 0.084 M, 4 eq.), HBTU (0.600 mL, 0.084 M, 4eq.) and DIPEA (0.300 mL, 0.168 M, 8 eq.) using the automatic dispenser contained in the synthesizer. The resulting mixture was then agitated for 20 s every 3 min for a total of 40 min. The reagents were removed by filtration under vacuum and the resin was washed with 4 aliquots of 1.5 mL DMF.

To achieve peptide synthesis, the above deprotection/coupling cycle was repeated until all the amino acids were coupled to the resin. Upon the completion of this stage of the synthesis, 3 mL of a cleavage solution comprising of TFA/TIPS/EDT/$H_2O$ (94:2.5:2.5:1 volume ratio) was added to the resin-loaded reaction syringe and the syringe was agitated for 3 h at room temperature. The cleavage solution was then removed from the syringe under vacuum and diethyl ether (10-15 mL) was added to precipitate the peptide. The resultant precipitate in diethyl ether was spun at 4°C and 4 000 rpm for 10 min to ensure pelleting the crude peptide. The diethyl ether supernatant was then decanted off and the peptide pellet was washed a further twice with diethyl ether. The crude peptide pellet was then re-dissolved in the minimum amount of water and freeze-dried using a Thermo Scientific Heto PowerDry LL1500 freeze-drier (Loughborough, UK) to remove any solvents. In order to form the disulfide bonds via aerial oxidation, the crude peptide was re-dissolved in water (1 mg 10 mL$^{-1}$) and stirred at room temperature for 7-10 days. The peptide solution was then concentrated and freeze-dried for storage prior to purification by HPLC.

**2.2.2 Purification and characterisation of the peptides**

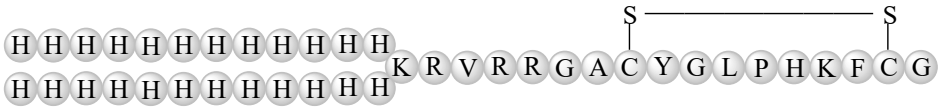
Peptides were purified and analysed via reverse phase HPLC using a Varian Prostar system equipped with a Model 210 solvent delivery module and a Model 320 UV detector (West Sussex, UK). Preparative HPLC purification was performed using a Phenomenex@ Onyx Monolithic Semi-Pre C18 column (100 x 10 mm, 2 µm macropore size, 13 nm mesopore size, Macclesfield, UK). The column was loaded with 200-400 µL aliquots of 10-20 mg mL$^{-1}$ of peptide dissolved in water containing 0.1% v/v of TFA. The mobile phase consisted of a decreasing gradient of water in acetonitrile ($CH_3CN$), both solvents contained 0.1% v/v of TFA. The precise experimental conditions, including mobile phase gradients, used are given below as Methods A and B.

**Preparative high performance liquid chromatography**:
**Method A**: Flow rate 9.5 mL/min; UV detection at 215 and 254 nm. Linear gradient: 5-25% B over 15 min. A = $H_2O$, 0.1% v/v of TFA, B = $CH_3CN$, 0.1% v/v of TFA.
**Method B**: Flow rate 9.5 mL/min; UV detection at 215 and 254 nm. Linear gradient: 5-30% B over 20 min. A = $H_2O$, 0.1% v/v of TFA, B = $CH_3CN$, 0.1% v/v of TFA.
The HLPC fractions containing the required peptide product were pooled and concentrated under reduced pressure to yield approximately 2 mL of solution prior to freeze-drying. The freeze-dried peptides were subsequently analysed by analytical HPLC using a Phenomenex@ Onyx Monolithic C18 column (100 x 3.0 µm, 2 µm micropore size, 13 nm mesopore size, Macclesfield, UK). The precise experimental conditions, including mobile phase gradients, used are given below as Methods C and D.

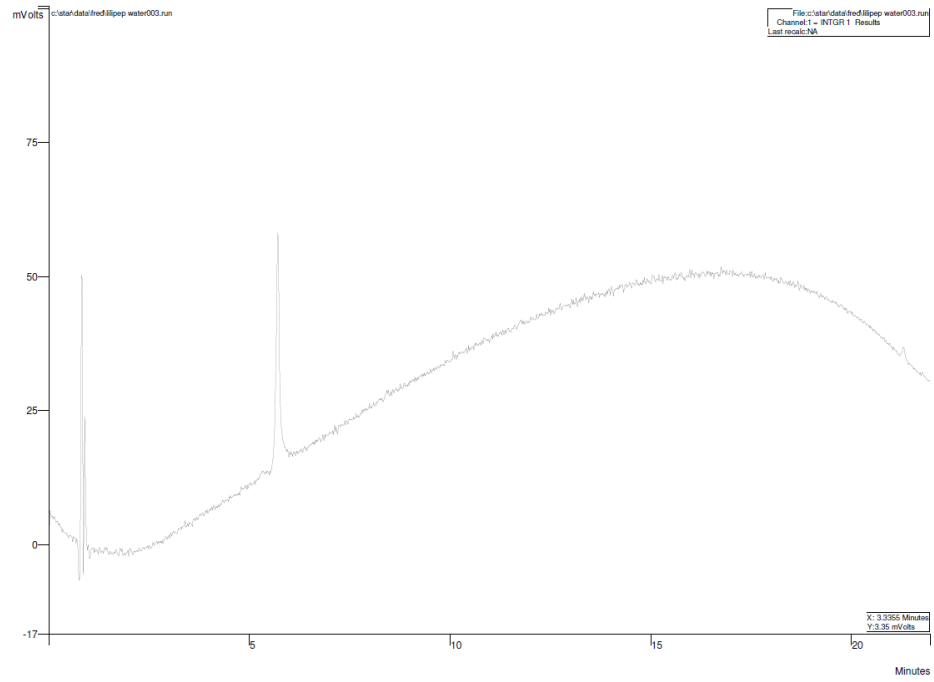
**Analytical high performance liquid chromatography**:
**Method C**: Flow rate 0.85 mL/min; UV detection at 215 and 254 nm. Linear gradient: 5-90% B over 20 min. A = $H_2O$, 0.1% v/v of TFA, B = $CH_3CN$, 0.1% v/v of TFA.
**Method D**: Flow rate 0.85 mL/min; UV detection at 215 and 254 nm. Linear gradient: 5-90% B over 30 min. A = $H_2O$, 0.1% v/v of TFA, B = $CH_3CN$, 0.1% v/v of TFA.
The analysis of the chromatograms was conducted using Star Chromatography Workstation software Version 1.9.3.2. ESI-MS analysis of the purified peptides was performed on a Waters Acquity Ultra Performance LC/MS machine (Elstree, UK).

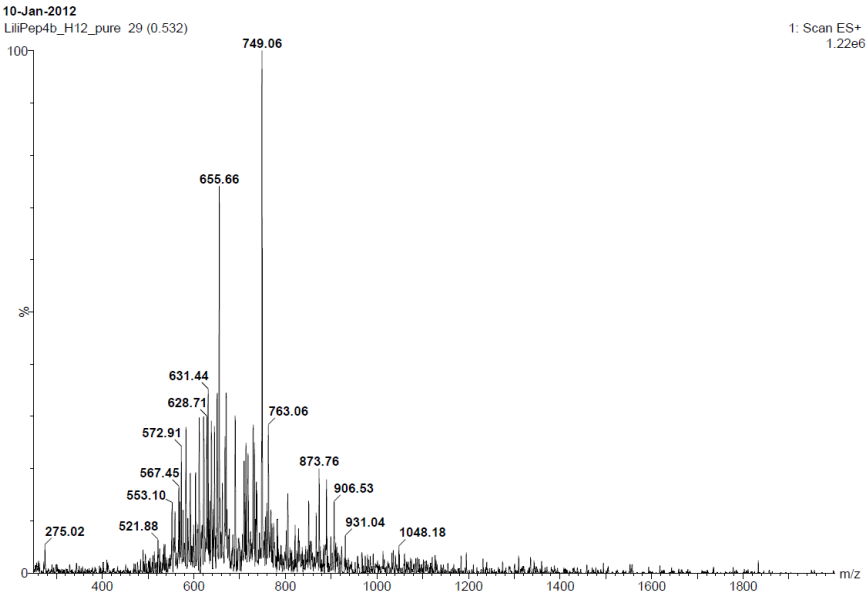
## HPLC analysis and mass spectra of peptides

### $H_{12}$BLY, MW 5238

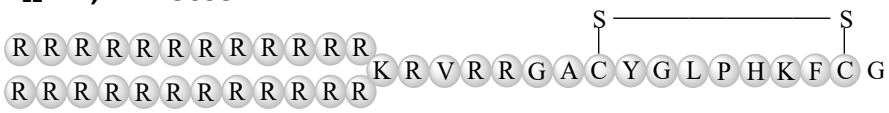



Purification was carried out using **Method B**. Analysis (**Method C**: $R_T$ = 5.715min) *m/z* (ES+) 1048.18([M+5H]$^{5+}$), 873.76([M+6H]$^{6+}$), 749.06([M+7H]$^{7+}$), 655.66([M+8H]$^{8+}$).

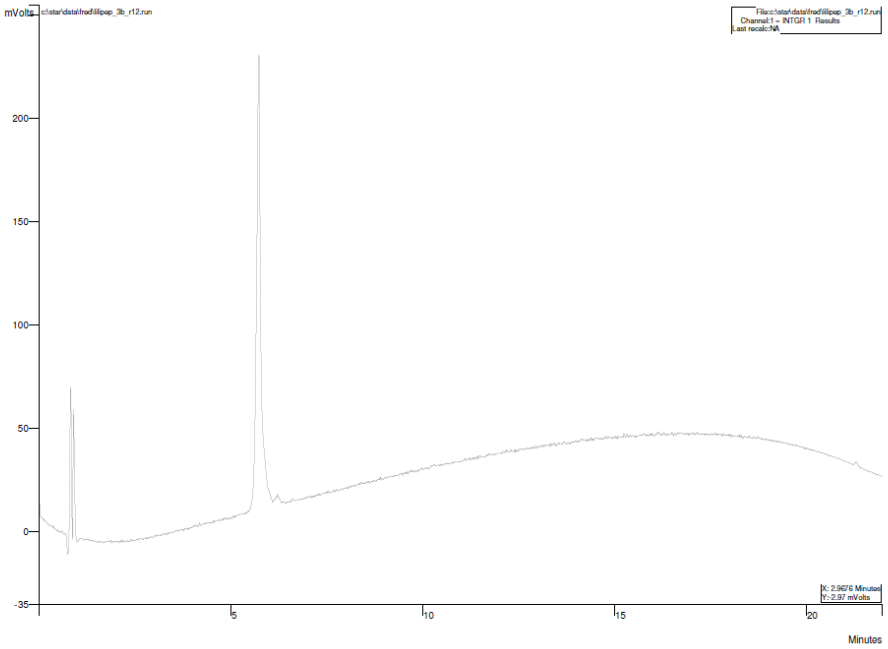


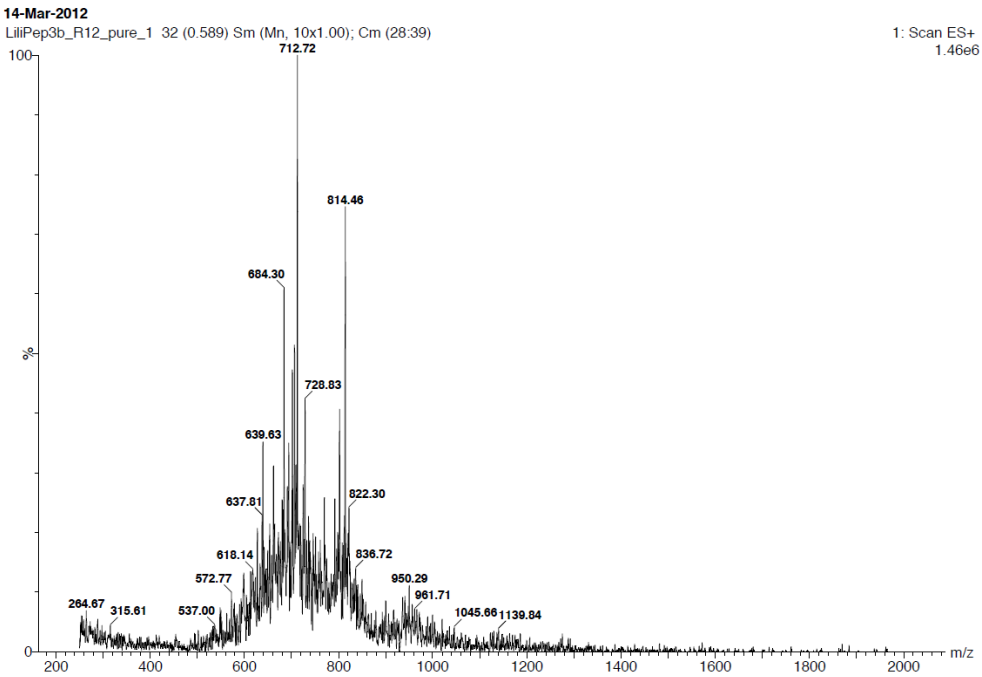



**$R_{12}$BLY, MW 5695**

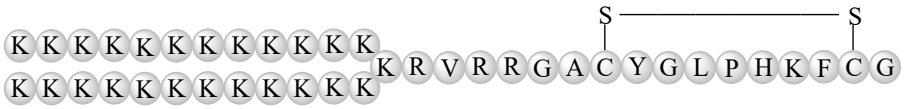



Purification was carried out using **Method B**. Analysis (**Method C**: $R_T$ = 5.71 min, *m/z* (ES+) 950.29([M+6H]$^{6+}$), 814.46([M+7H]$^{7+}$), 712.72([M+8H]$^{8+}$).

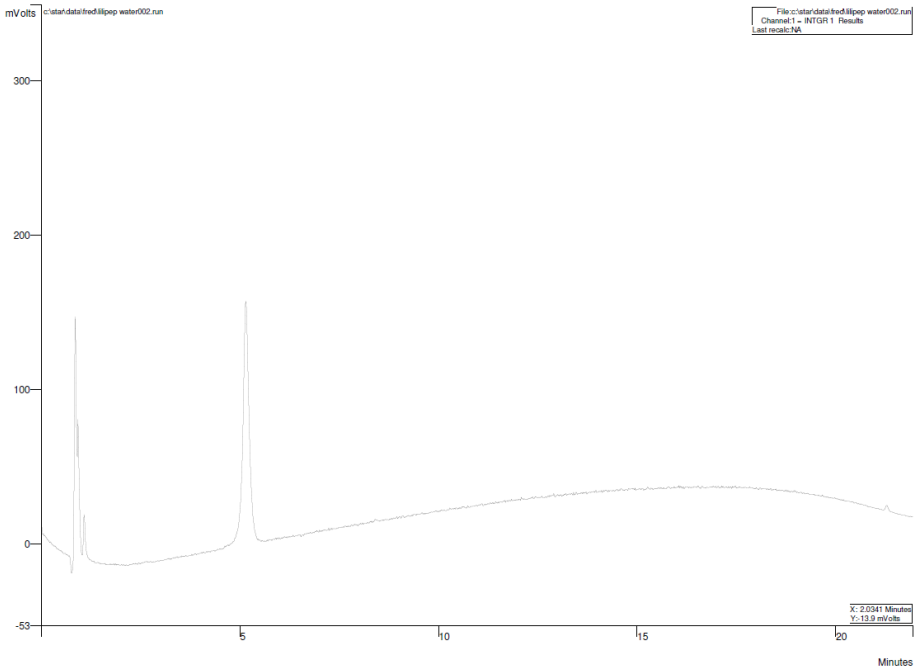


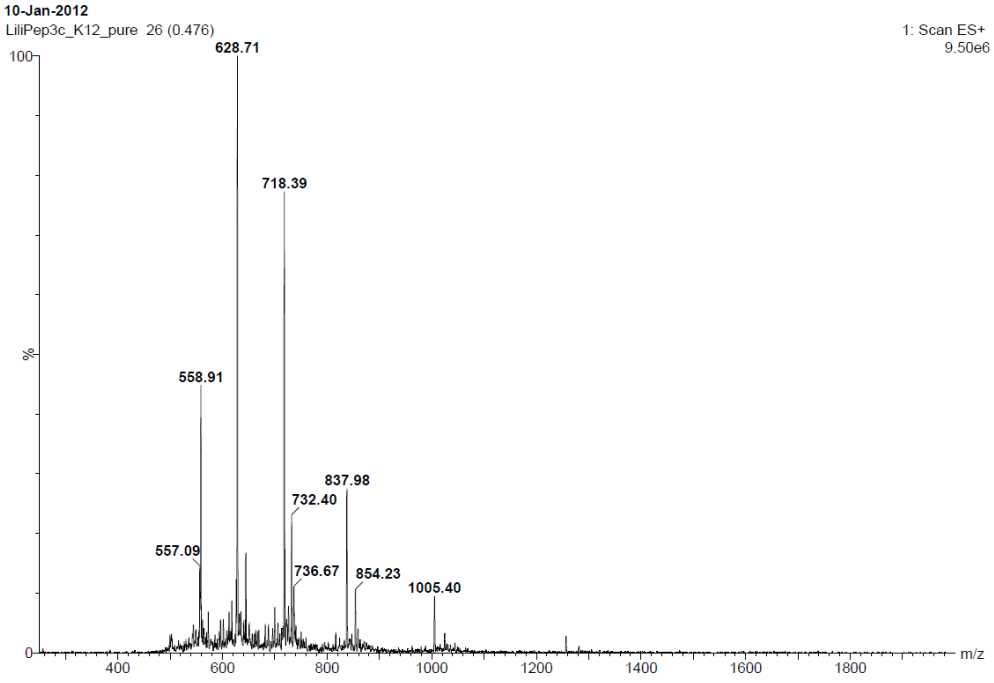



**$K_{12}$BLY MW 5022**

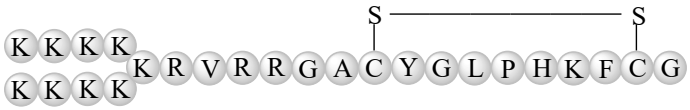



Purification was carried out using **Method B**. Analysis (**Method C**): $R_T$ = 5.147min, *m/z* (ES+) 1005.40([M+5H]$^{5+}$),837.98([M+6H]$^{6+}$), 718.39([M+7H]$^{7+}$), 628.71([M+8H]$^{8+}$), 558.91([M+9H]$^{9+}$).

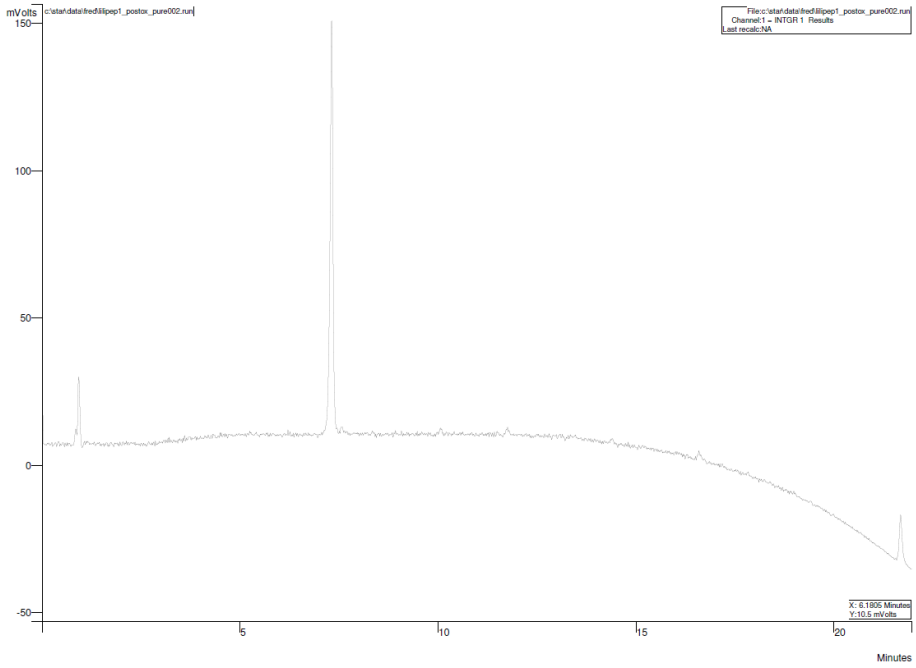


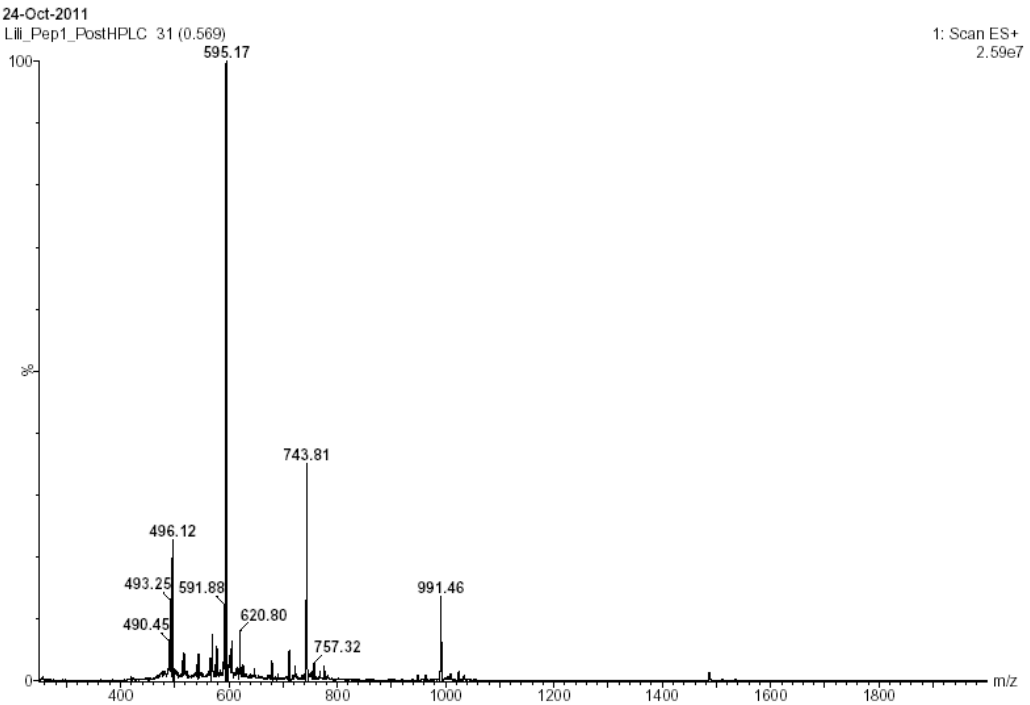



**$K_4$BLY MW 2972**

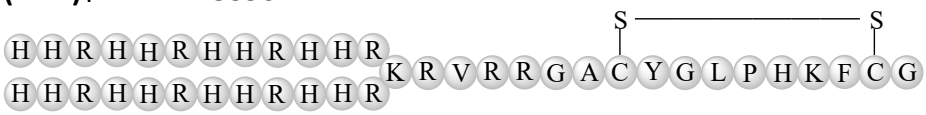



Purification was carried out using **Method A**. Analysis (**Method C**): $R_T$ = 7.3086min, *m/z* (ES+) 743.74 ($[M+4H]^{4+}$), 595.17($[M+5H]^{5+}$), 496.05($[M+6H]^{6+}$).

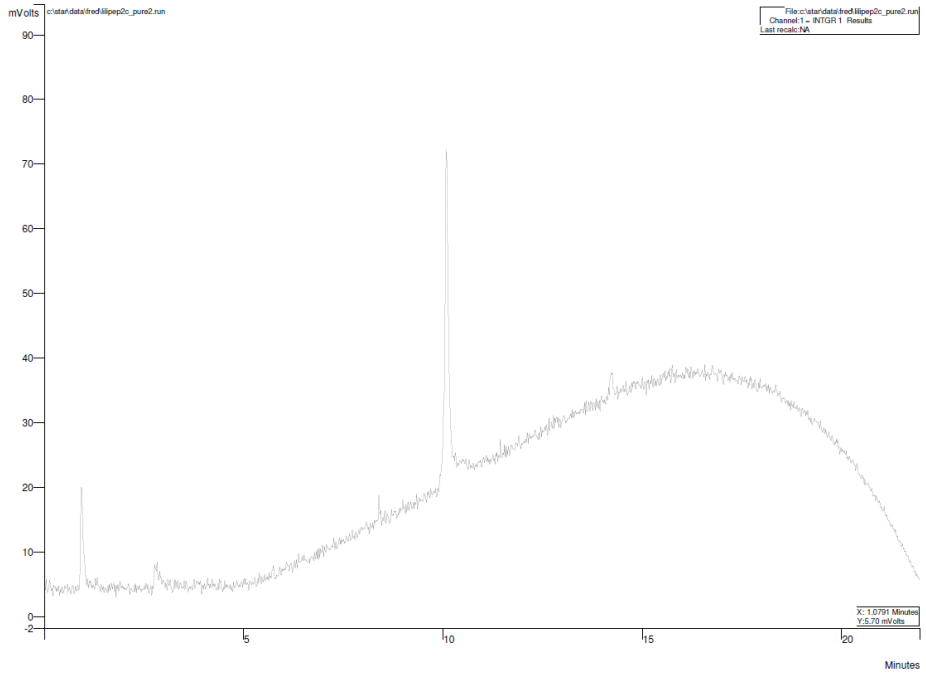


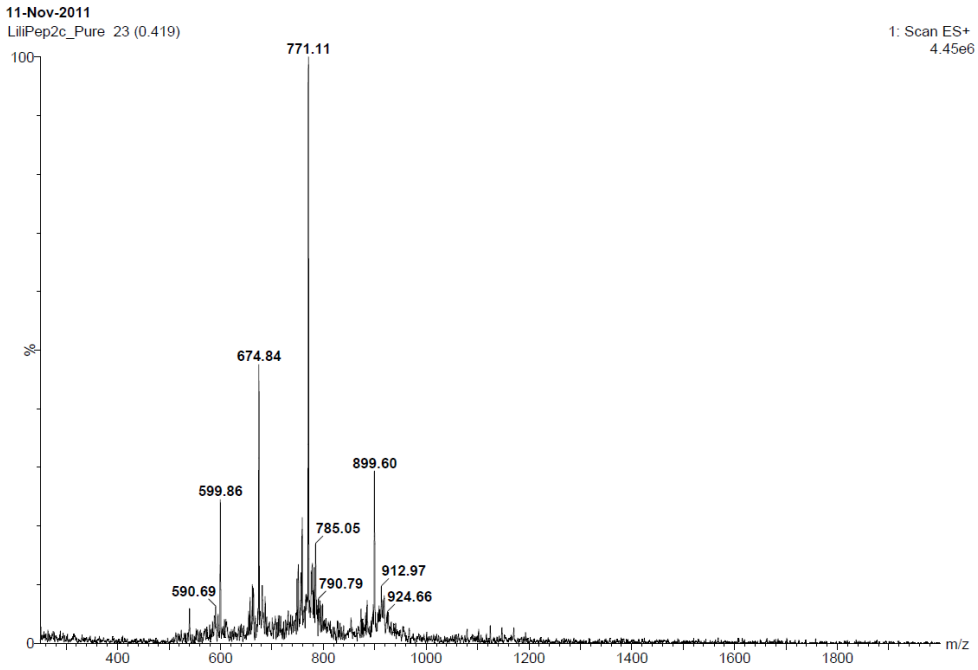



**(HHR)$_4$BLY MW 5390**

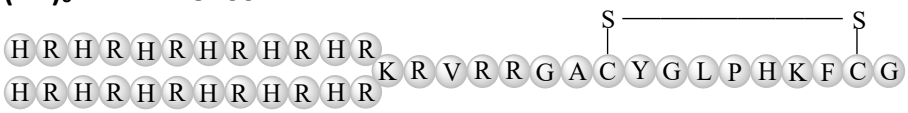



Purification was carried out using **Method A**. Analysis (**Method C**): $R_T$ = 10.129min, *m/z* (ES+) 899.60([M+6H]$^{6+}$), 771.11([M+7H]$^{7+}$), 674.84([M+8H]$^{8+}$), 599.86([M+9H]$^{9+}$).

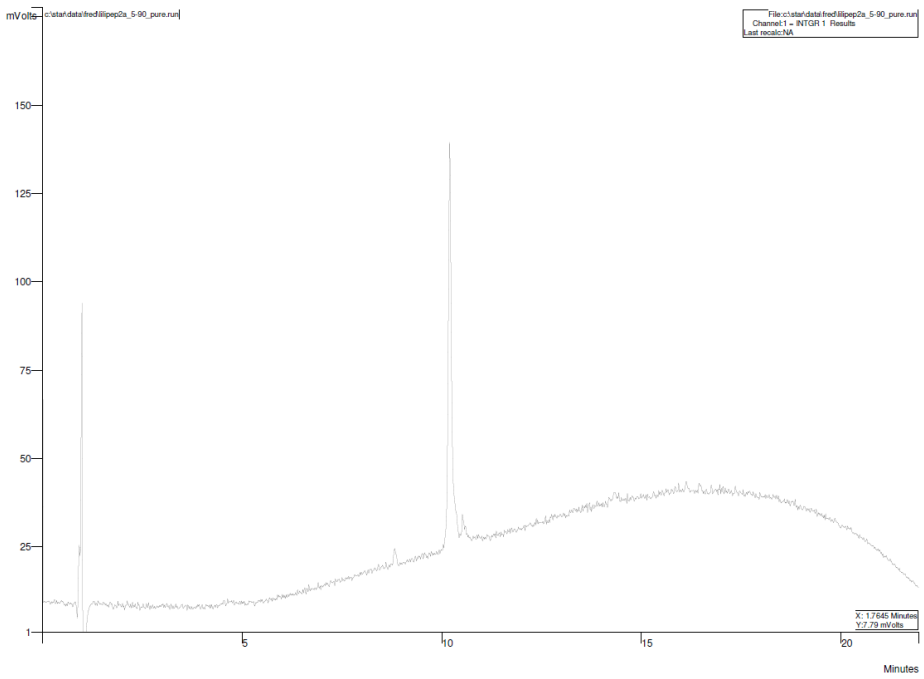


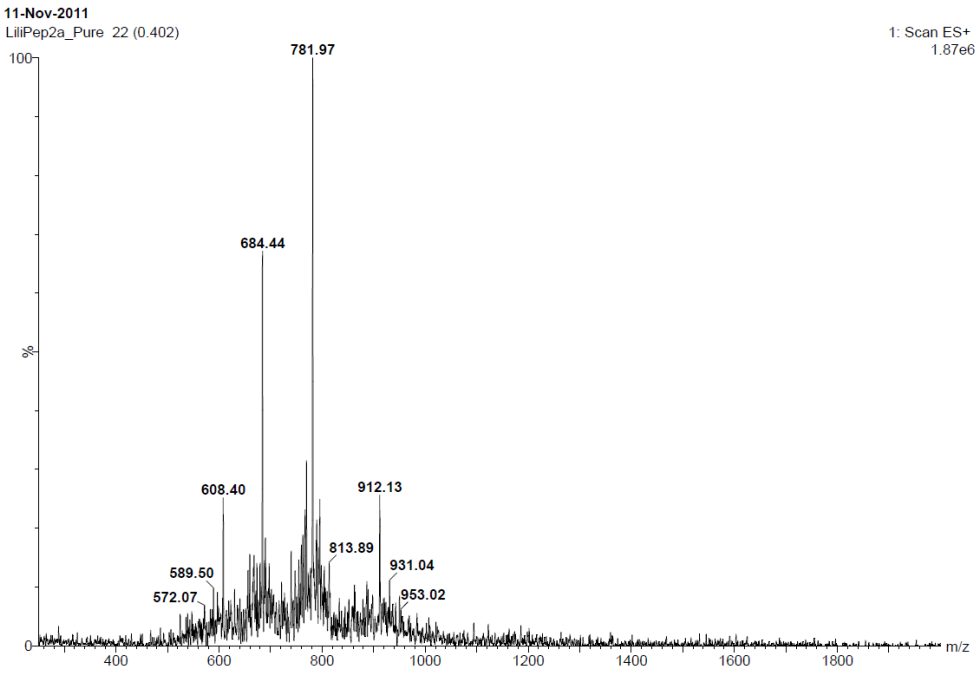



**$(HR)_6$BLY MW 5466**

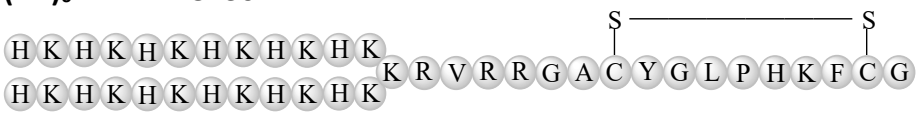



Purification was carried out using **Method A**. Analysis (**Method C**): $R_T$ =10.195min, *m/z* (ES+) 912.13 ($[M+6H]^{6+}$), 781.97 ($[M+7H]^{7+}$), 684.44($[M+8H]^{8+}$), 608.40($[M+9H]^{9+}$).

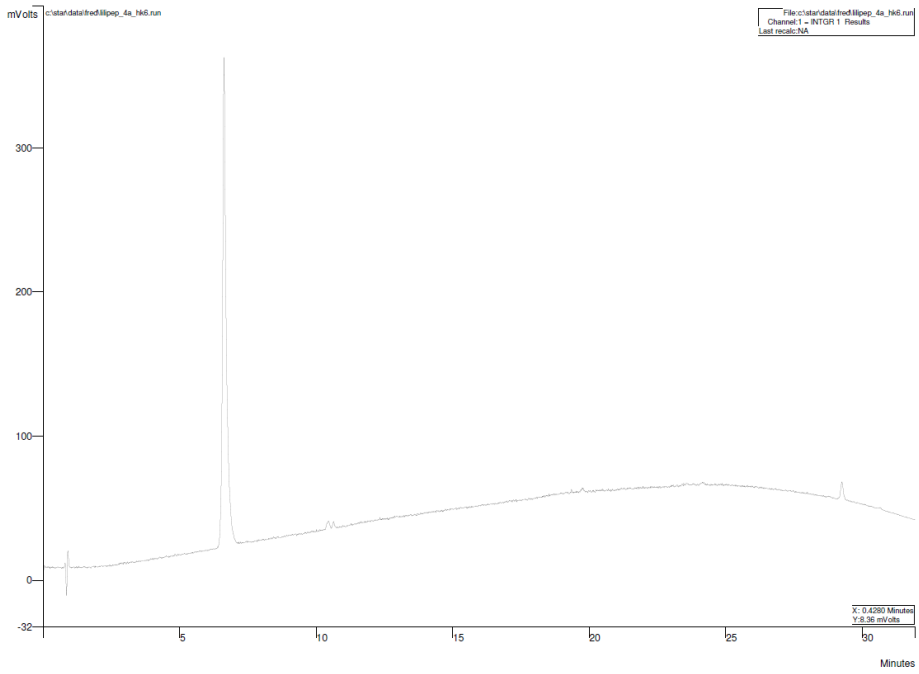


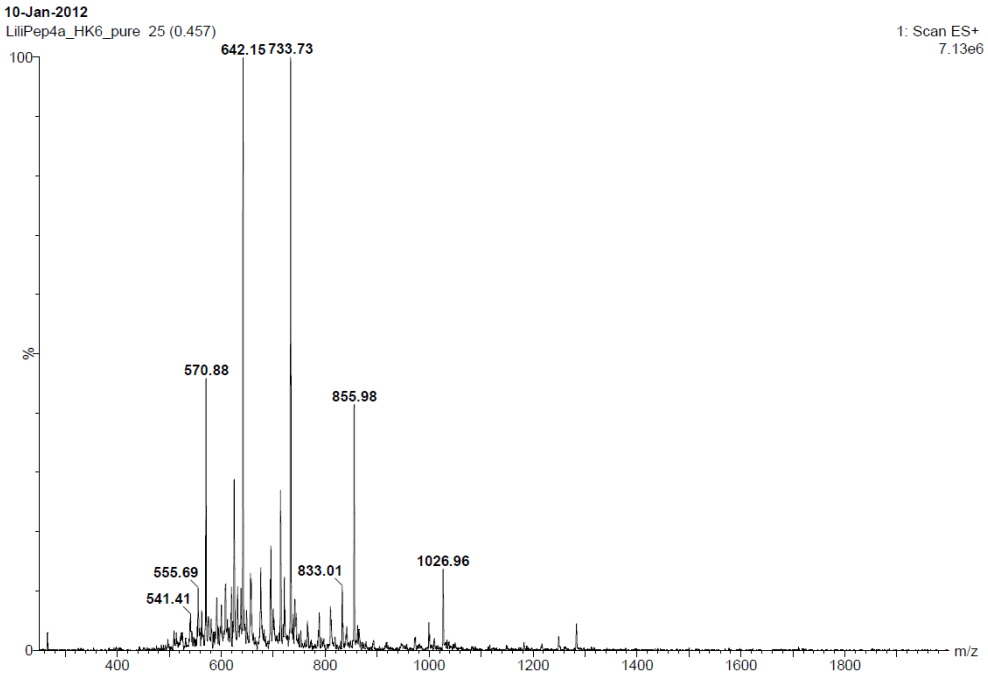



**$(HK)_6$BLY MW 5130**

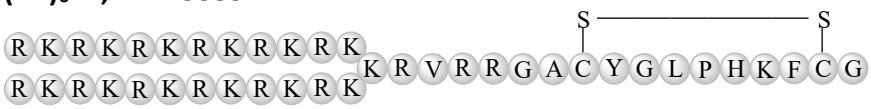



Purification was carried out using **Method B**. Analysis (**Method D**): $R_T$ = 6.630min, *m/z* (ES+) 1026.96([M+5H]$^{5+}$),855.98([M+6H]$^{6+}$), 733.73([M+7H]$^{7+}$), 642.15([M+8H]$^{8+}$), 570.88([M+9H]$^{9+}$).

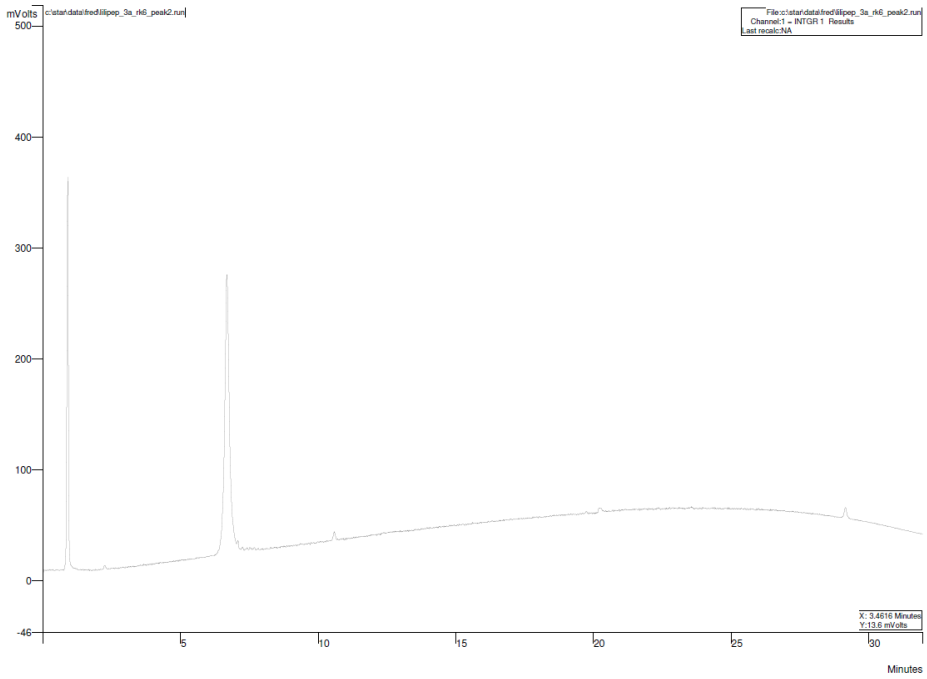


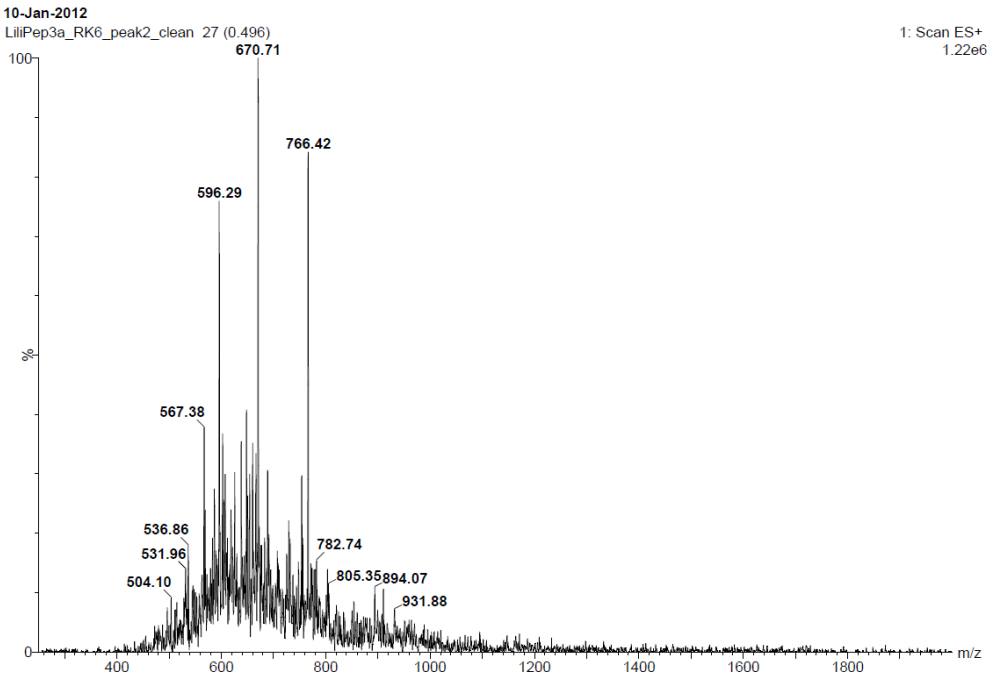



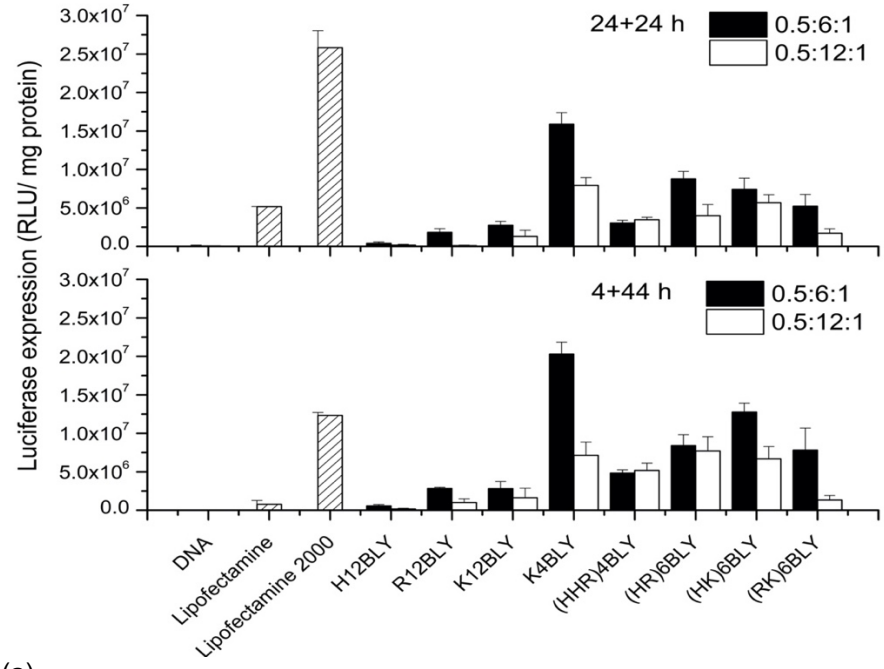
**(RK)$_6$BL, MW 5359**



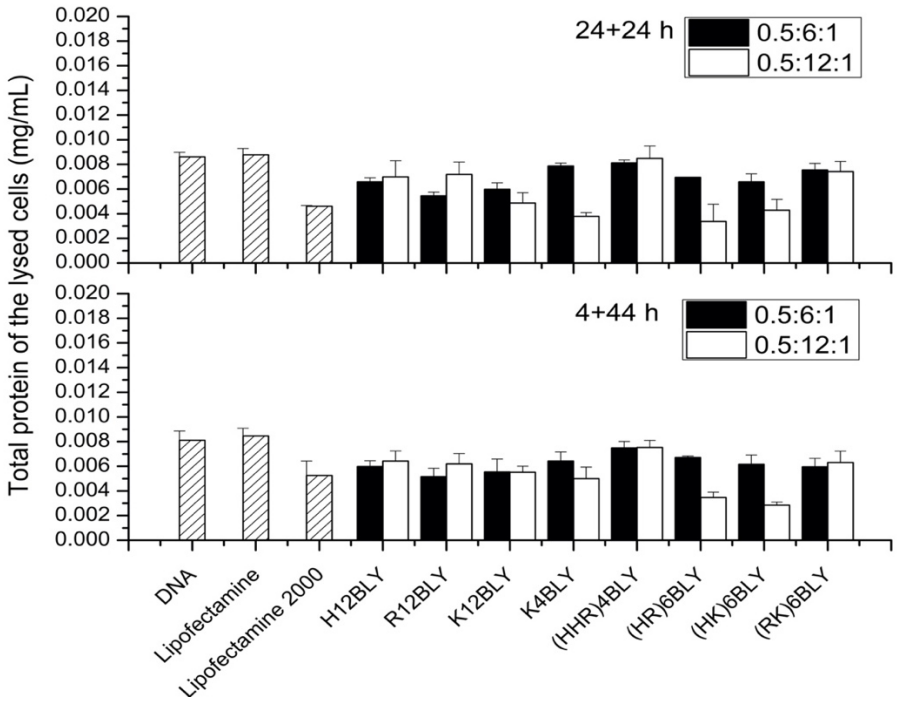

Purification was carried out using **Method B**. Analysis (**Method D**): $R_T$ = 6.690min, *m/z* (ES+) 910.66([M+6H]$^{6+}$), 766.42([M+7H]$^{7+}$), 670.71([M+8H]$^{8+}$), 596.29([M+9H]$^{9+}$), 536.86([M+10H]$^{10+}$).



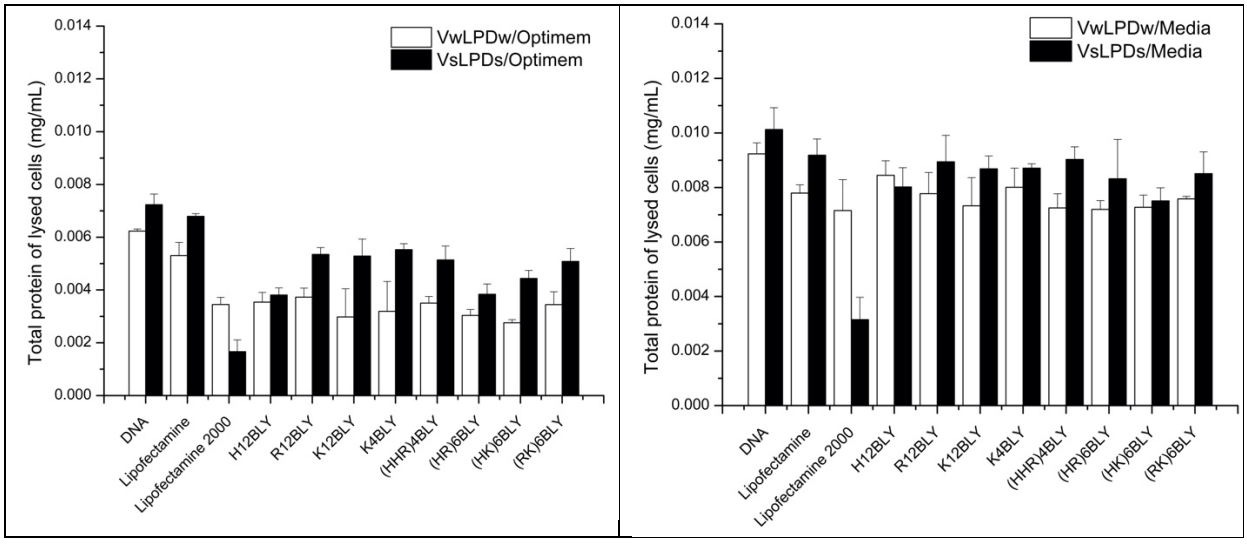



(a)




(b)

**Figure S1** Transfection efficiency of various VwLPDw in A549 cells in the presence of OptiMEM (a) and corresponding protein content assay (b). Vw indicates that lipid vesicles used to make LPD were prepared in water and LPDw mean that LPD complexes were prepared in water. A549 cells were transfected for either 24 h or 4 h. In all samples, the lipid:peptide:DNA charge ratio was kept at 0.5:6:1 and cationic vesicles were composed of DOTMA/DOPE at 1:1 molar ratio. Data are mean values ± standard deviation of three replicates.




(a) (b)

**Figure S2** Protein content assay in A549 cells after exposure to VwLPDw and VsLPDs in the presence of Optimem (a) RPMI-1640 medium containing 10% FBS (b). Vw and Vs indicate that lipid vesicles used to make LPD were prepared in water and 120 mM NaCl solution, respectively. LPDw and LPDs mean that LPD complexes were prepared in water and 120 mM NaCl solution, separately. The incubation time of LPD with A549 cells was 4 h. Cationic vesicles used to prepare the LPDs were composed of DOTMA:DOPE at 1:1 molar ratio. Data are mean values ± standard deviation of three replicates.

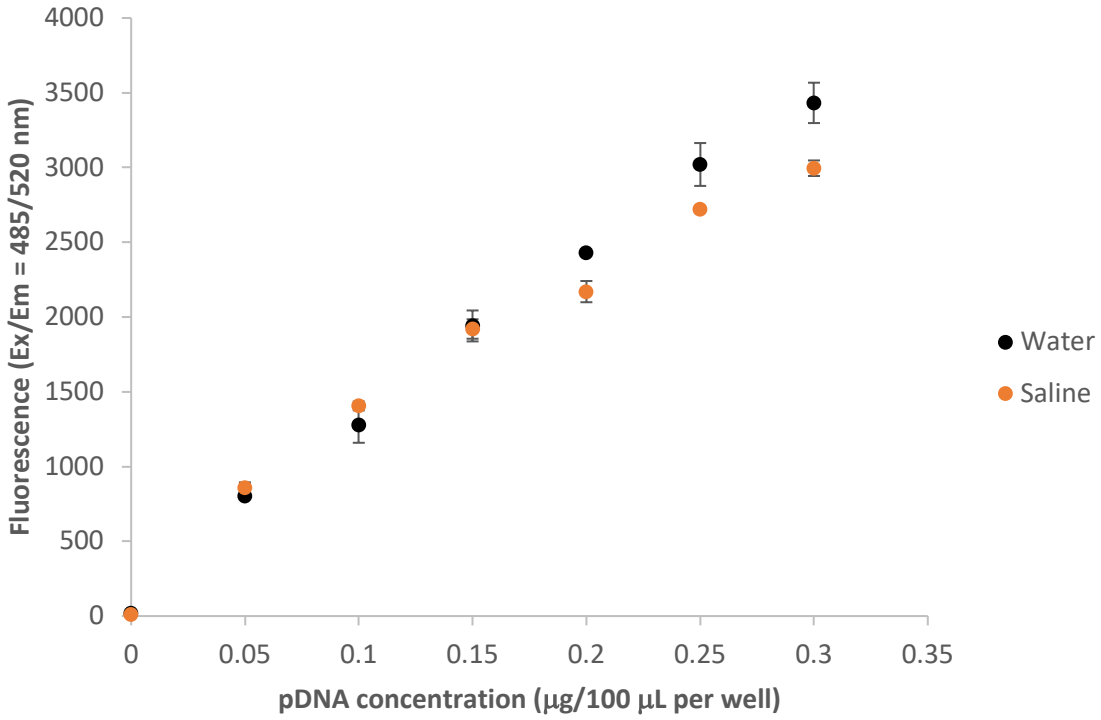
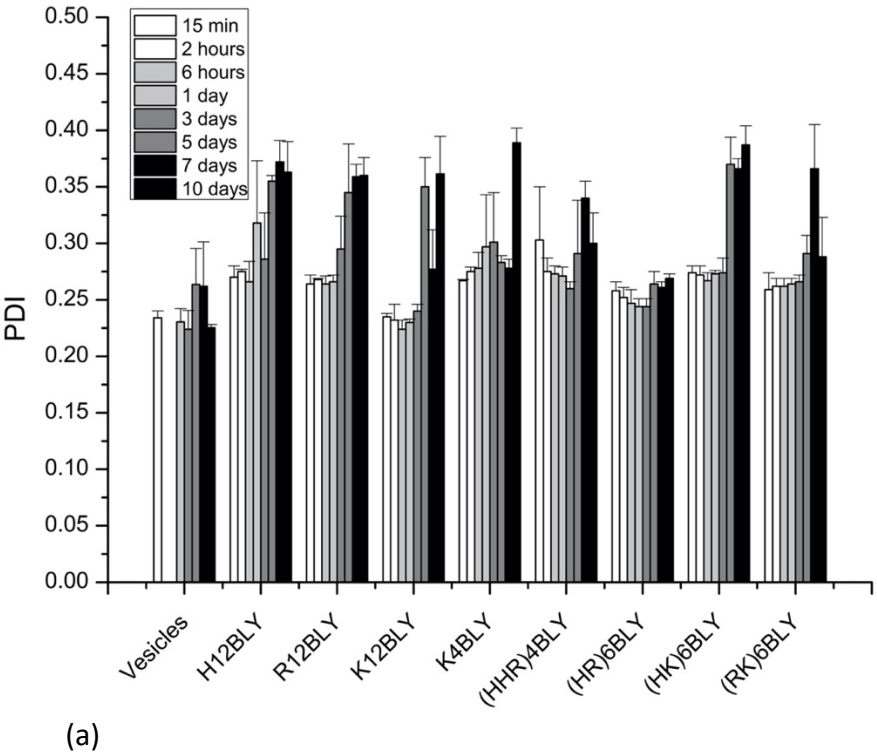
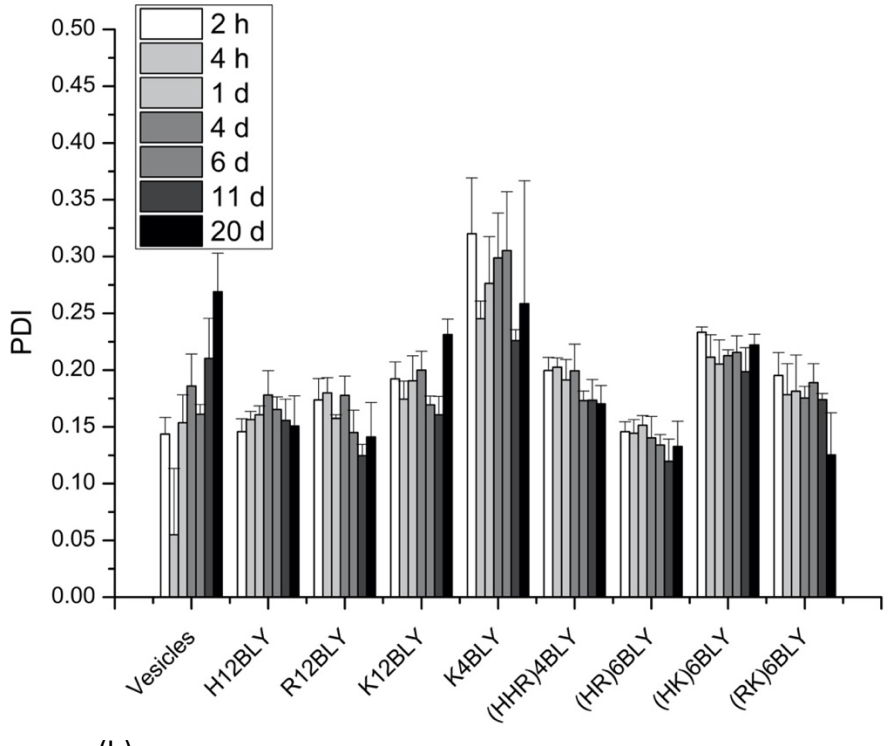



**Figure S3:** Picogreen calibration graphs of pDNA prepared either in water (black circles) or 120 mM NaCl solution (red circles). Data are mean values ± standard deviation of three replicates. A linear relationship is observed in water-containing samples at all concentrations tested. However, the fluorescence of picogreen-DNA samples prepared in NaCl starts tailing off above 0.15 μg/100 μL, hence 0.1 μg/100 μL of pDNA was used in NaCl-containing samples in subsequent experiments.




(a)




(b)

**Figure S4** Polydispersity of LPDs made in (a) water (VwLPDw) and (b) 120 mM NaCl solution (VsLPDs). In all samples, the lipid:peptide:DNA charge ratio was kept at 0.5:6:1 and 0.5:12:1, respectively (final DNA concentration of 0.01 mg/mL). Cationic vesicles were composed of DOTMA/DOPE at 1:1 molar ratio. Measurements were performed at 25 ± 0.1°C (n=3). Data are mean values ± standard deviation of three replicates.