Supporting Information for

Designed Filamentous Cell Penetrating Peptides: Probing Supramolecular Structure-Dependent Membrane Activity and Transfection Efficiency

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General Methods.

MBHA rink amide resin, Fmoc-protected amino acids, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were purchased from Novabiochem. Piperidine was purchased from Sigma-Aldrich. All other reagents and solvents for peptide synthesis and purification were purchased from Fisher Scientific and used as received. Desalting column VariPure IPE was ordered Agilent Technologies (Apple Valley, MN). Glo lysis buffer and luciferase assay kit were purchased from Promega (Madison, WI). BCA protein assay kit was obtained from Thermo Scientific (Waltham, MA). Plasmid DNA encoding a 5.2 kb firefly luciferase (pCMV-luc) was purchased from Elim Biopharmaceuticals (Hayward, CA). Label IT® fluorescein labelling kit was purchased from Mirus Bio (Madison, WI, USA). Dulbecco's modified Eagle medium (DMEM) culture medium was purchased from Life Technologies. Fetal Bovine Serum (FBS) was ordered from VWR. CCK8 assay kit was obtained from Dojindo Molecular Technologies (Rockville, MD). Circular dichroism (CD) spectra were acquired on a Jasco-J815 spectrometer using a quartz cell with 1 mm path length. Transmission electron microscopy (TEM) was conducted on a JEOL 2010 high-resolution transmission electron microscope. Reversed-phase HPLC was carried out using HITACHI L-7100 pump, Waters 717 plus auto-sampler and Higgins analytical column (proto 300 C4 10 μm, 250*10 mm). Luminescence and UV absorbance were measured on a micro-plate reader (Vitor^2 1420 Multilabel Counter, PerkinElmer) for transfection and toxicity quantification.

Synthesis and Purification of Peptides

The synthesis of MDPs followed the standard Fmoc-solid phase peptide synthesis method. Briefly, Fmoc group was deprotected in 20% (V/V) piperidine/DMF for 5 minutes and repeated once. HCTU was used as the coupling reagent and the coupling reaction was carried out in the presence of diisopropyl ethyl amine (DIPEA) for 45 mins. Fmoc protected amino acids were added in five equivalents of the resin for each coupling step. Upon completion of the synthesis, the N-terminus of the MDPs was acetylated in the presence of acetic anhydride and DIPEA in DMF for a duation of 1 hr. The completion of

the reaction was confirmed by the Kaiser test. The acetylated peptide was cleaved from the resin with a mixture of TFA / triisopropanolsilane (TIS) / H_2O (95/2.5/2.5 by volume) for 3 hours. Cleavage solution was collected and the resin was rinsed twice with neat TFA. TFA was evaporated with air blow and the residual peptide solution was triturated with cold diethyl ether. The resulting precipitate was centrifuged at 6500 rpm for 5 min and washed with cold diethyl ether for three times. Crude peptide was then dried under vacuum overnight for further HPLC purification. Peptides were purified using a preparative reverse phase C18 column with a linear gradient of binary water/acetonitrile mixed solvent containing 0.05% TFA. Elution was monitored at 280 nm. The HPLC fraction was collected, combined and subject to desalting to remove the trifluoroacetates in the peptide solution. The desalted HPLC fraction was frozen in liquid nitrogen and placed in the lyophilizer for 3 days. The molecular weight of the peptides was confirmed by ESI-MS. For $K_{10}(QW)_6$, expected $[M+H]^+$: 3225.80, observed MW:3226.10; For $K_5(QW)_6K_5$, expected $[M+H]^+$: 3225.80, observed MW: 3226.87; For $K_6(QW)_6$, expected $[M+H]^+$: 2713.40, observed MW:2712.34.

Circular Dichroism (CD) Spectroscopy

MDPs were diluted in Tris buffer (pH 7.4, 20 mM) to reach a final concentration of 100 μM for CD measurements. The data were collected from 250 nm to 190 nm at room temperature with a scan rate of 100 nm/min, a response time of 2 sec and a bandwidth of 1 nm. The final spectra were an average of five scans. The mDeg of rotation directly from the instrument was converted to molar residual ellipticity via the formula θ=(mDeg*1000)/(c*n*l), where c is the concentration of the peptide solution expressed in mM, n is the number of amino acids in the peptide sequence and l is the path length of the cell used in mm.

TEM

MDPs were diluted in Tris buffer (pH=7.4, 20 mM) to reach a final concentration of 100 μM. 10 μl of the sample solution was dropped onto a holey carbon grid (TED PELLA 01824). After 1 min, the excess solution was carefully removed with filter paper and the

sample was stained by adding 10 µl of 2 wt% uranyl acetate solution for 1 min. The excess staining solution was removed with filter paper and the TEM sample was allowed to dry for overnight before imaging. For the preparation of peptide-DNA complex, MDPs were diluted in Tris buffer (pH=7.4, 20 mM) to reach a final concentration of 640 µM. Then 10µL of peptide solution was mixed with equal volume of DNA (0.108mg/mL) to reach a N/P ratio of 20:1. The solution was incubated for 1 hr at room temperature. 10 µl of the solution was dropped onto a holey carbon grid (TED PELLA 01824). After 1 min, the excess solution was carefully removed with filter paper and the sample was stained with 10 µl of 2 wt % uranyl acetate solution for 1 min. The excess staining solution was removed with filter paper and the TEM sample was allowed to dry for overnight before imaging.

Ethidium Bromide (EB) Exclusion Assay

Peptides, DNA and EB were diluted in Tris buffer (pH=7.4, 20 mM) to the desired concentration for mixing as described below. 5 µl of DNA (0.216 mg/mL) solution was added in a 48-well plate, followed by the addition of peptides to reach the desired N/P ratio. The solution was incubated for 15 min at room temperature to form stable complex. 5 µl of EB (0.05 mg/mL) solution was added in each well and incubated for another 15 min. Fluorescence emission of the mixture was measured at λ_{ex}=520 nm and λ_{em}= 600 nm. Pure EB solution and DNA/EB complex without peptides were used as negative (F_0) and positive (F_{EB}) controls, respectively. The exclusion efficiency (%) was defined as: $(F-F_0)/(F_{EB}-F_0)$ x100

Protection Against Enzymatic Degradation

Agarose gel electrophoresis was performed using a 15 well Bio-Rad Mini-Sub® Cell GT system. Gels were prepared using 1% (w/v) agar in 1X TAE buffer with an ethidium bromide concentration of 0.5 µg/mL. N/P ratios were calculated based on the charge of the peptide monomer to the charge of the phosphate groups in a single nucleic acid strand. Peptide powder was suspended and diluted with 20 mM Tris buffer (pH 7.4). Samples were prepared by sequentially adding 20 mM Tris buffer, luciferase, and then peptides at

N/P ratios 2, 5, 10, and 15 for a final [DNA] = 10 μg/ml. Samples equilibrated at room temperature for 30 minutes and then either DNase I (0.2 U), trypsin (final 0.1 mg/ml), or FBS (final 10%) was added to the samples for a final [DNA]= 9.1 μg/ml. Enzymes and serum was supplemented with 20 mM Tris buffer for negative controls and positive controls contained enzymes and serum without peptide. After 30 minute incubation at RT, 5 μl DNase Stop Solution (50 mM EDTA) was added to stop the enzymatic reaction. The complexes were mixed with 1X agarose gel loading dye and electrophoresed in 1X TAE buffer at 100 V for 15 min. The gels were imaged with using a UV transilluminator.

Dynamic Light Scattering

Dynamic light scattering (DLS) was performed on a Malvern Zetasizer. N/P ratios were calculated based on the charge of the peptide monomer to the charge of the phosphate groups in a single nucleic acid strand. Peptides and luciferase DNA were diluted with 20 mM Tris buffer (pH 7.4). Samples were prepared by adding equal volumes of peptide and DNA solution at N/P 1, 2, 5, 10 and 20. The samples equilibrated and were then measured by DLS. A minimum of 6 scans was taken for each sample.

Cell Uptake of Hoechst 33258

The membrane activity of MDPs was evaluated by monitoring the fluorescence of DNA-bound membrane-impermeable dye, Hoechst 33258. Hoechst 33258 was dissolved in DMSO and diluted in Tris buffer (20 mM, pH 7.4) as the stock solution. HeLa cells were seeded in a confocal dish at a density 5×10^4 cells/well and incubated for 24 hrs. The medium was replaced with fresh DMEM containing 10% deactivated FBS. Hoechst 33258 was added in the cell culture media to reach a final concentration of 3 μM. After 5 mins, MDPs or MDP/DNA (N/P=10) complexes was added to the cell culture and the fluorescence was recorded in real time. The final concentration of MDP is 16 μM for $K_{10}(QW)_6$ and $K_5(QW)_6$ K_5. The fluorescence images were processed by Image J and plotted by using Igor Pro 6.04.

Cell uptake of FITC-labeled luciferase gene/MDP complexes

HEK293 and HeLa cell lines were used to investigate the cellular uptake of $K_{10}(QW)_6$ and $K_5(QW)_6K_5$/DNA complexes. Luciferase plasmids were labeled using Label IT® fluorescein labelling kit following the protocol provided by the manufacture. Cells were seeded onto a 24-well plate at a density of 1 x 10^5 cells/well and cultured for 24 hrs. DMEM medium was replaced and peptide-DNA complex solutions were added with a final peptide concentration at 16 μM and the N/P ratio at 10. After incubation with peptide/DNA complexes for 2 hrs, cells were washed with PBS buffer containing heparin (20 U/mL) for 3 times. Thereafter, the cells were harvested with trypsin, washed twice with PBS buffer containing heparin. Then cells were fixed using 2% paraformaldehyde for 10 min. Cell uptake of FITC-labeled luciferase/peptide complexes was quantified using a BD FACS Caliburs flow cytometer. A minimum of 10,000 events per sample was analyzed. The fluorescence signal was analyzed using the FITC-A channel. Data was processed using FlowJo software.

In vitro Cytotoxicity

For cytotoxicity measurement, HEK293 and HeLa cells were seeded into a 96-well plate with a density of 10000 cells/well and incubated for 24 hrs. Luciferase plasmid was mixed with MDPs in an equal volume to reach N/P ratio at 10 in Tris buffer. DNA-peptide complexes were added (DNA 0.54 μg/well) in the cell culture and incubated for 4 hrs. The media were removed and replaced with fresh DMEM containing 10 % deactivated FBS and incubated for 20 hrs. Cell viability was determined by using CCK-8 assay according to the manufacturer's protocol. The optical density of each well was determined on a microplate reader (Vitor^2 1420 Multilabel Counter, PerkinElmer, USA) at the wavelength of 450 nm. All the experiments were performed in six replicates.

Gene Transfection

HEK 293 and Hela cells were cultured in DMEM with 10 % deactivated FBS under 5 % of CO_2 at 37℃. The culture medium was changed every two days. For transfection measurement, HEK293 cells and Hela cells were seeded into a 96-well plate at a density of 10000 cells/well and incubated for 24 hrs. The DNA-peptide complex at the N/P ratio of 10 was incubated at room temperature for 1 hr to form stable complex. The DNA-peptide complex (DNA 0.54 μg/well) were added in the cell culture and incubated for 4 hrs. The media was removed and replaced with new DMEM containing 10% deactivated FBS for 20 hrs of incubation. Culture media was removed and 50 μL of 1×Glo lysis buffer was added. After two cycles of freezing (-80 °C for 30 min) and thawing (room temperature), cell lysates were centrifuged at 2000 rpm for 15 min to remove cell debris. The supernatant was isolated and luciferase expression was determined according to the manufacturer's protocol. The relative light unit (RLU) reading from the luminometer was normalized by the protein concentration in the supernatant, which was determined by BCA protein assay. All the experiments were performed in six replicates.

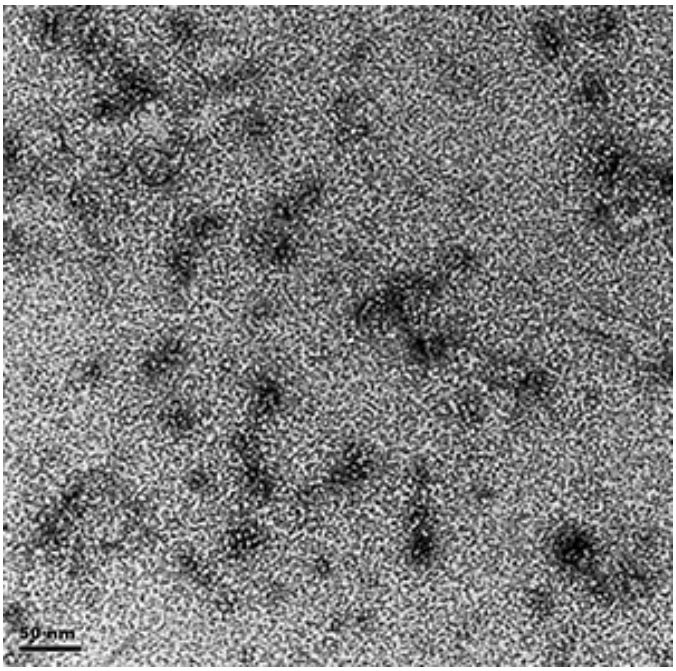

Figure S1. Negatively stained TEM image of $K_5(QW)_6K_5$ at 100 μM forming irregular aggregates.

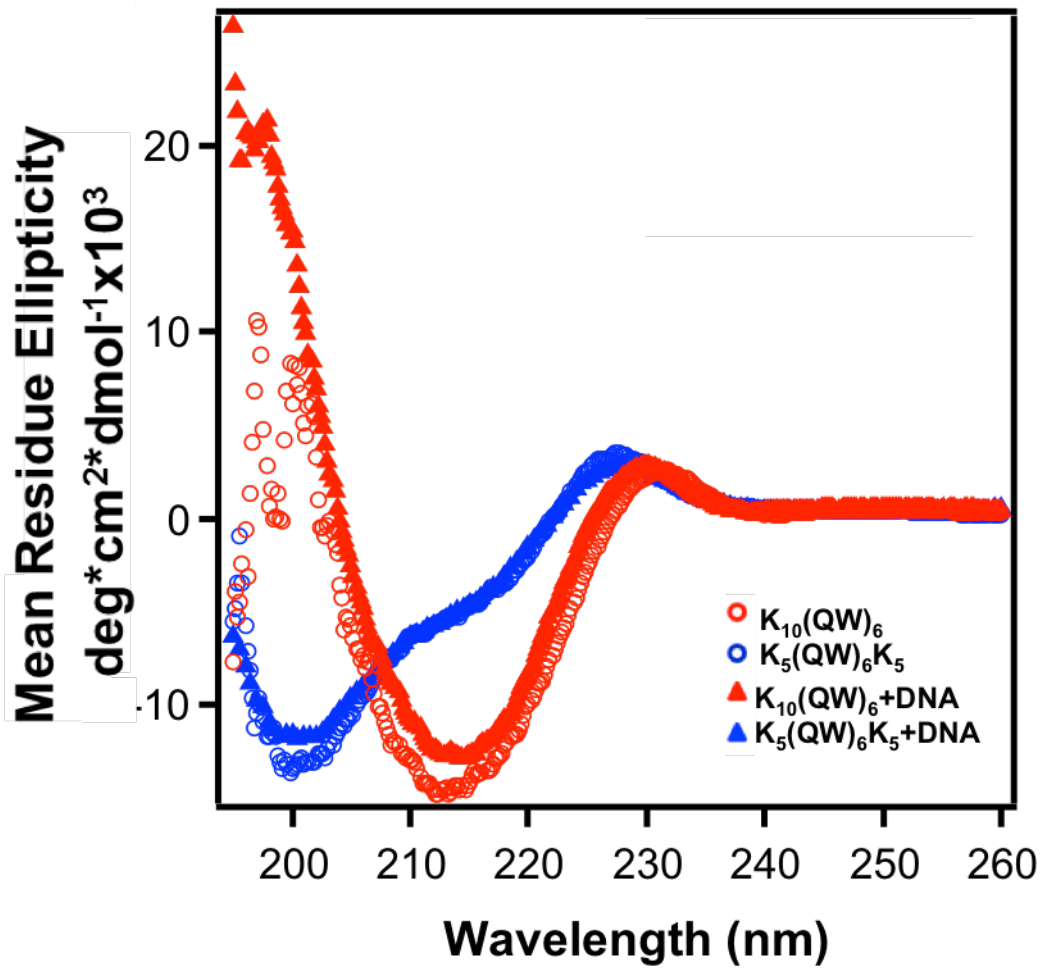

Figure S2. CD spectra of $K_{10}(QW)_6$, $K_5(QW)_6K_5$ and peptide/DNA complexes at a N/P ratio of 10. Peptide concentration: 100 μM in Tris buffer (20 mM, pH =7.4). The secondary structures of the complexes remain the same as those of peptides alone with minimum peak shift.

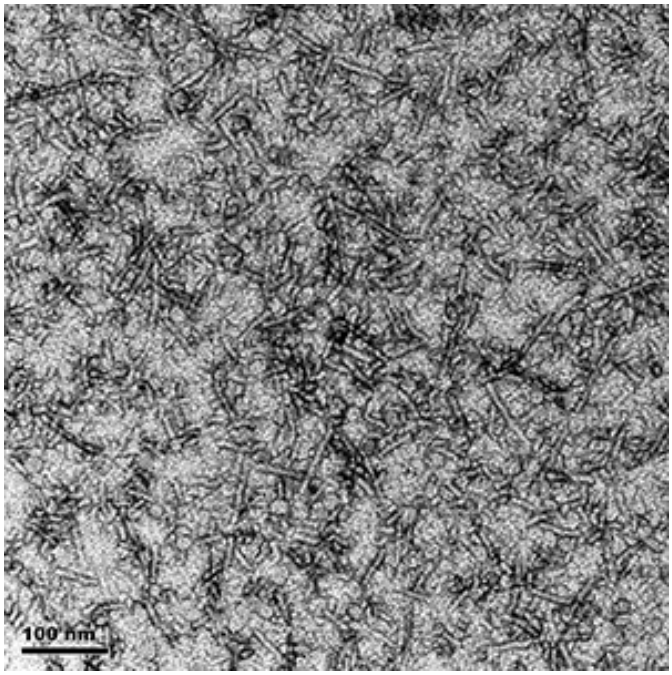

Figure S3. Negatively stained TEM image of $K_6(QW)_6$ at 100 μM in Tris buffer (pH=7.4 20 mM)

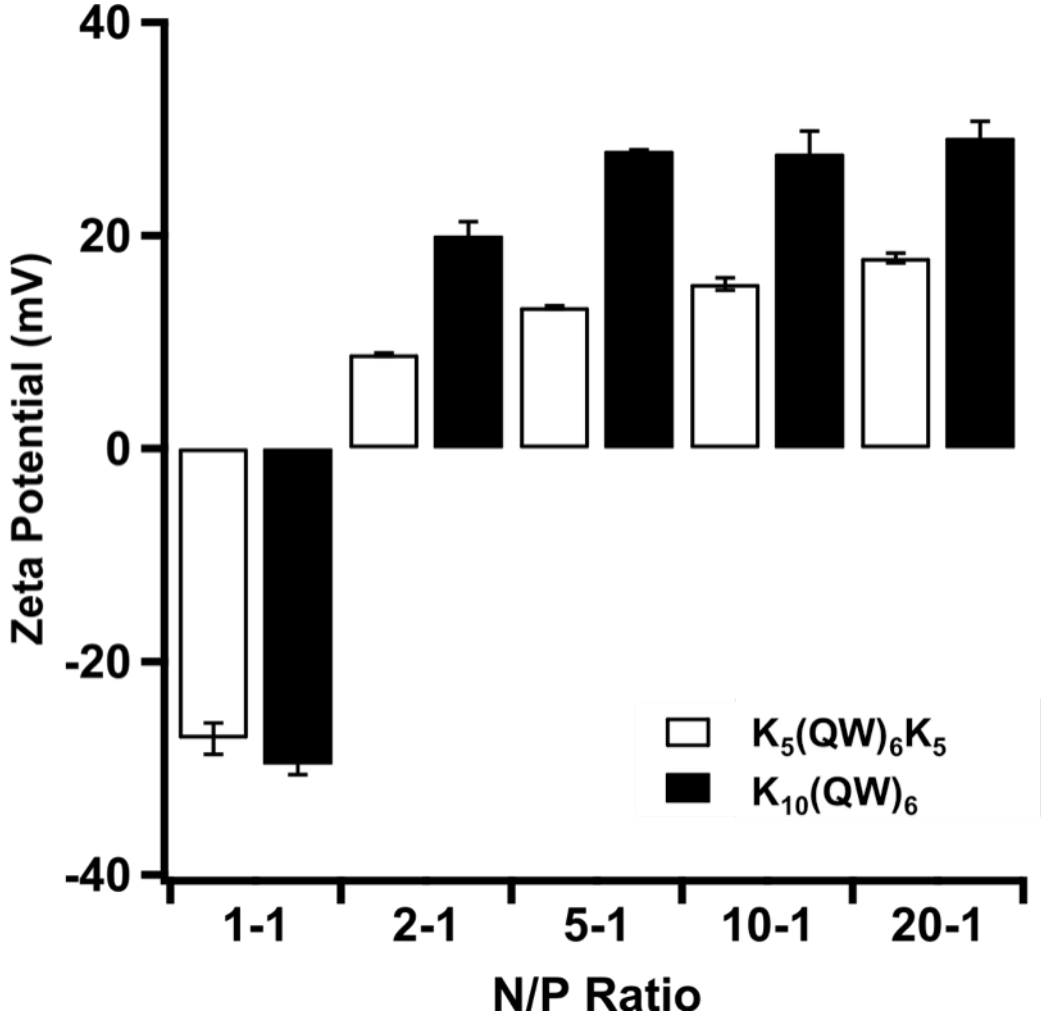

Figure S4. Zeta potential measurements of peptide/DNA complexes at varying N/P ratios with three replicates for each sample measurement.

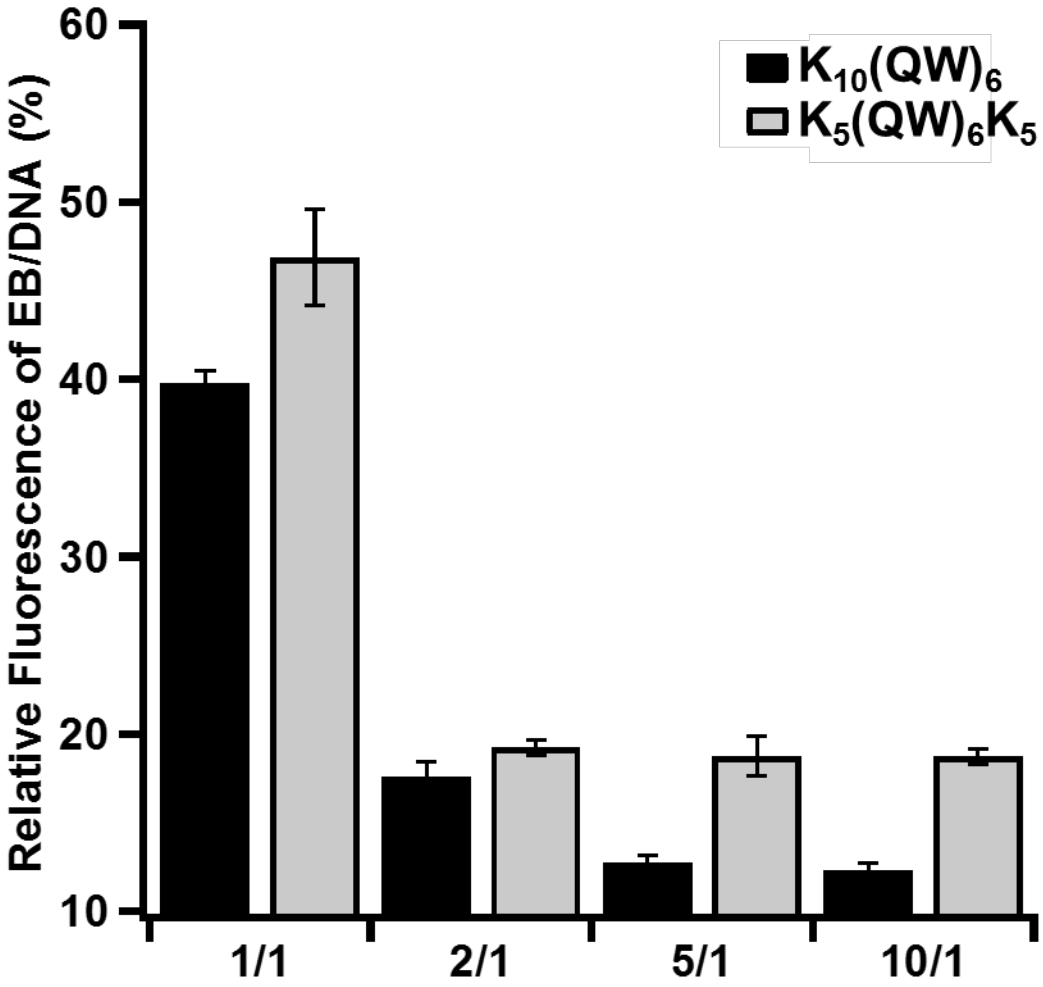

Figure S5. The efficiency of EB exclusion for different MDP-DNA complexes. The relative fluorescence intensity reflects the accessibility of EB to DNA.

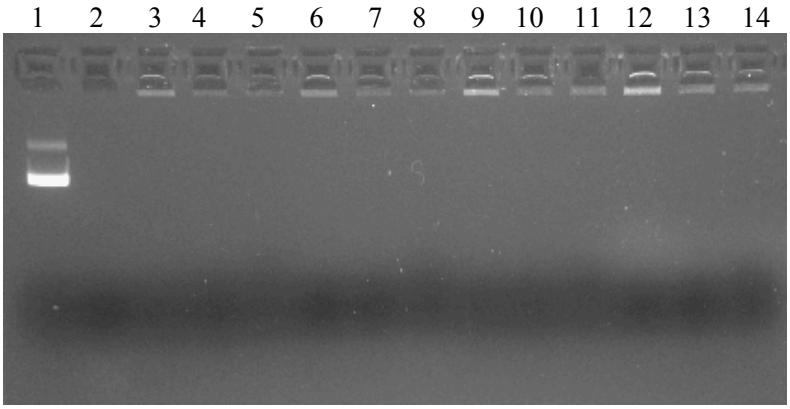

Figure S6. Agarose gel showing the stability of peptide and plasmid complexes incubating with DNase I. Lane **1**: DNA plasmid. Lane **2**: DNA plasmid treated with DNase. Lane **3** to **5**: $K_{10}(QW)_6$/DNA with N/P ratio of 2, 5, 15 respectively. Lane **6** to **8**: $K_{10}(QW)_6$/DNA with N/P ratio of 2, 5, 15 respectively in the presence of DNase. Lane **9** to **11**: $K_5(QW)_6K_5$/DNA with N/P ratio of 2, 5, 15 respectively. Lane **12** to **14**: $K_5(QW)_6K_5$/DNA with N/P ratio of 2, 5, 15 respectively in the presence of DNase.

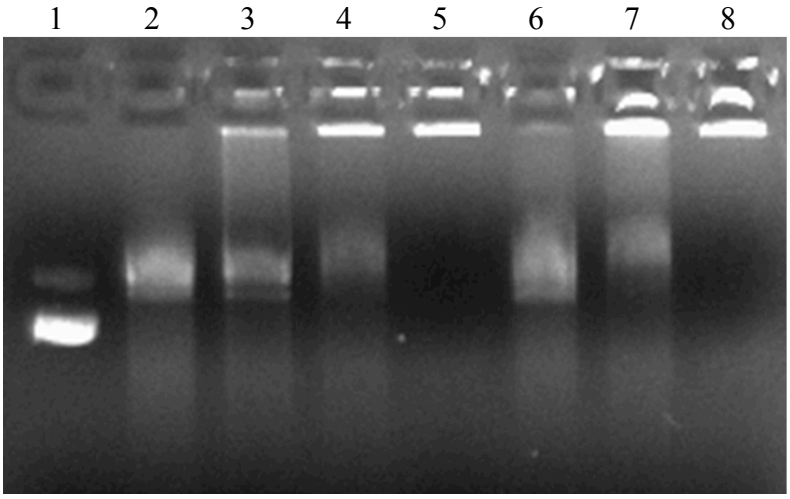

Figure S7. Agarose gel showing the stability of peptide and plasmid complexes incubating with FBS. Lane **1**: DNA plasmid. Lane **2**: DNA plasmid treated with FBS. Lane **3** to **5**: $K_{10}(QW)_6$/DNA with N/P ratio of 2, 5, 10 respectively in the presence of FBS. Lane **6** to **8**: $K_5(QW)_6K_5$/DNA with N/P ratio of 2, 5, 10 respectively in the presence of FBS.

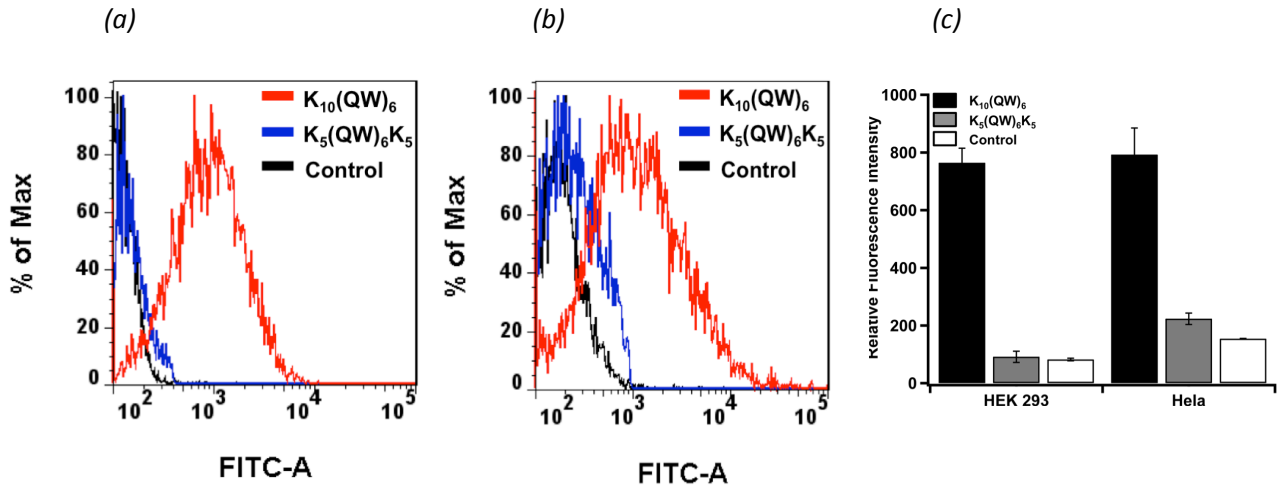

Figure S8. Flow cytometry evaluation of cell uptake of peptide/luciferase gene complexes in (a) HEK 293 cells (b) HeLa Cells (c) Quantification of the fluorescence intensity of internalized FITC-labeled DNA/peptide complex.

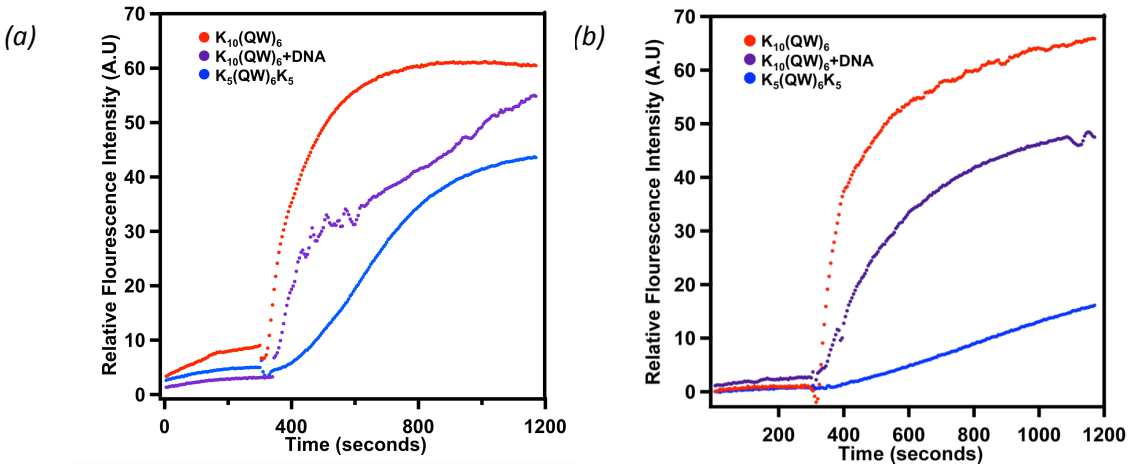

Figure S9. Kinetics of Hoechst 33258 uptake facilitated by peptides alone and peptide/DNA complexes in (a) HEK 293 and (b) HeLa cells. Plot of Hoechst 33258 uptake by $K_5(QW)_6K_5$/DNA complex was not successful due to the interference of the background fluorescence of the peptide/DNA complex upon binding with Hoechst 33258. The real-time movie can clearly distinguish the localization of the Hoechst 33258 bound to the $K_5(QW)_6K_5$/DNA complex in the culture medium rather than being internalized inside cells.

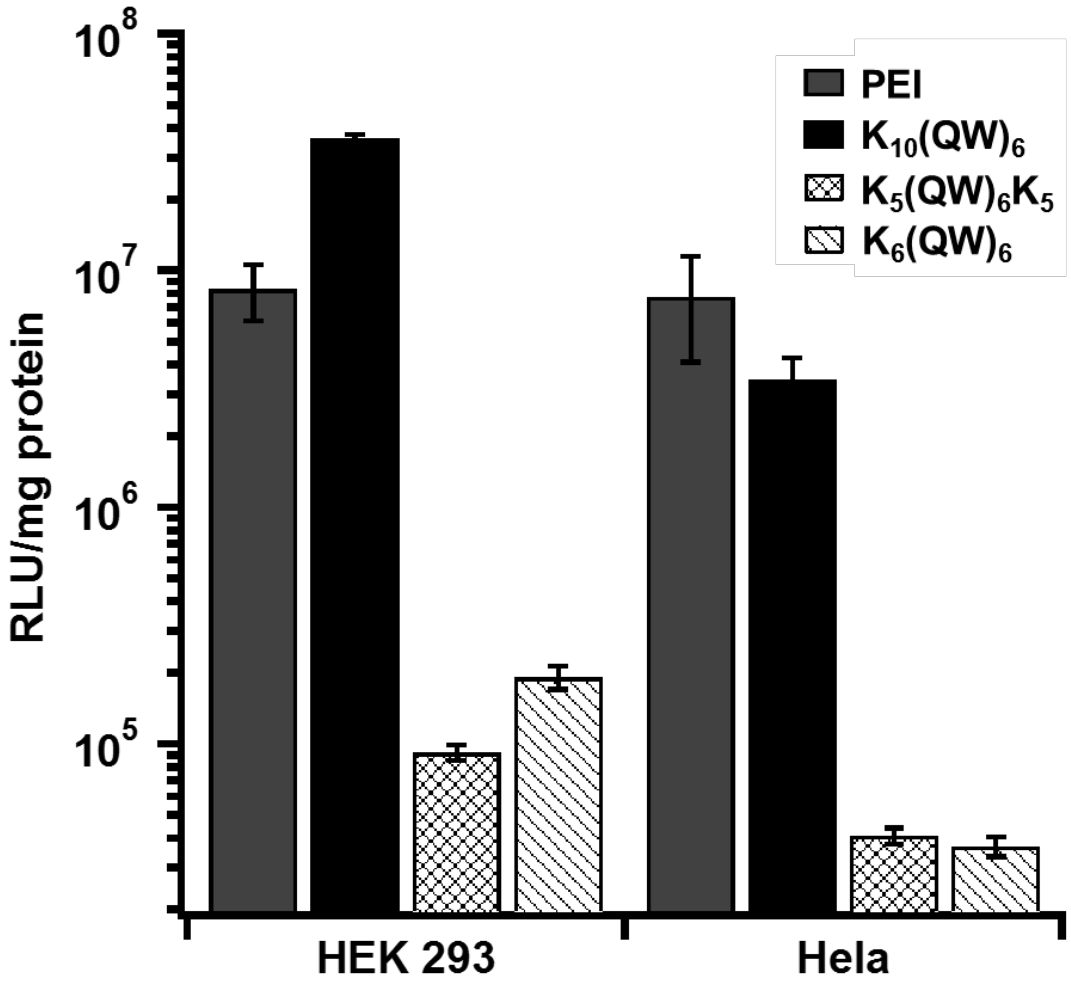

Figure S10. Transfection efficiency of MDPs and PEI in both HEK293 and HeLa cell lines. N/P ratio = 10. Luciferase gene/per well=0.54 μg. All the measurements were performed in six replicates.

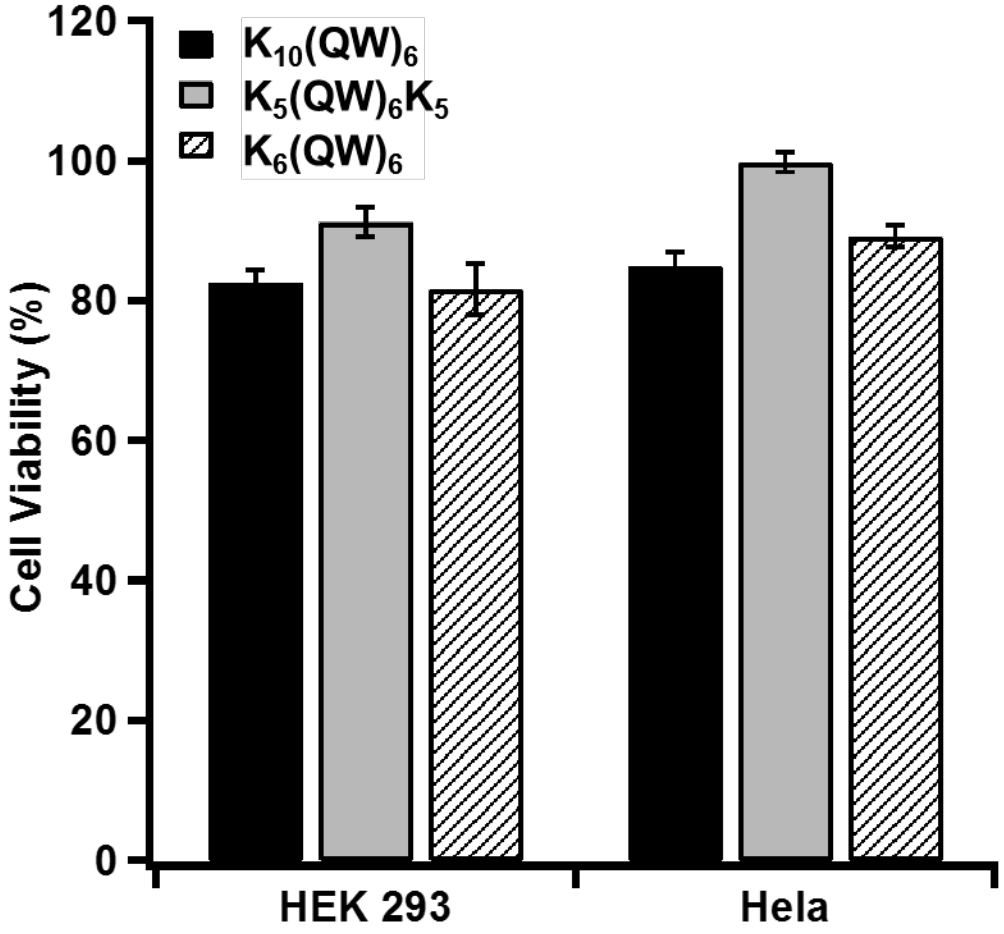

Figure S11. Cytoxocity evaluation of MDPs-plasmid complex toward HEK293 and HeLa cells. N/P ratio=10 with luciferase gene per well at 0.54 μg. All the measurements were performed in six replicates.

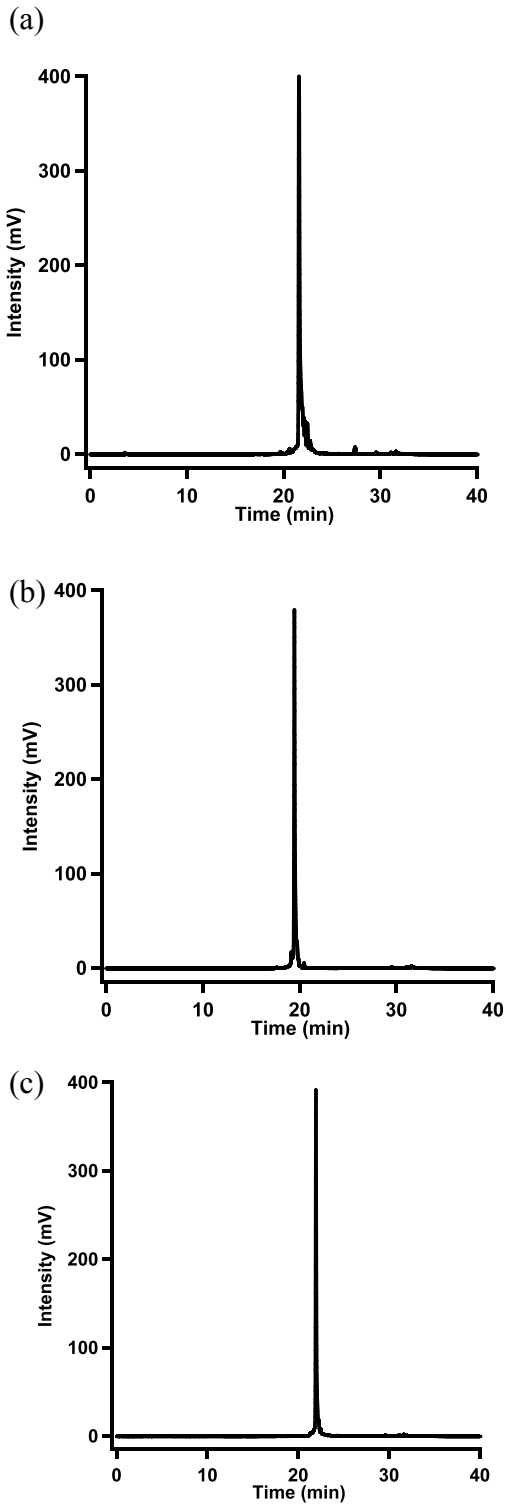

Figure S12. Analytical HPLC spectra of (a)$K_{10}(QW)_6$, (b)$K_5(QW)_6K_5$ and (c) $K_6(QW)_6$ showing the purify of all the peptides used in the study is > 95%.