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Incorporation of a nuclear localization signal in pH responsive LAH4-L1 peptide enhances transfection and nuclear uptake of plasmid DNA

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ABSTRACT

The major intracellular barriers associated with DNA delivery using non-viral vectors are inefficient endosomal/lysosomal escape and poor nuclear uptake. LAH4-L1, a pH responsive cationic amphipathic peptide, is an efficient DNA delivery vector that promotes the release of nucleic acid into cytoplasm through endosomal escape. Here we further enhance the DNA transfection efficiency of LAH4-L1 by incorporating nuclear localizing signal (NLS) to promote nuclear importation. Four NLSs were investigated: Simian virus 40 (SV40) large T-antigen derived NLS, nucleoplasmin targeting signal, M9 sequence and the reverse SV40 derived NLS. All peptides tested were able to form positively charged nano-sized complexes with DNA. Significant improvement in DNA transfection was observed in slow-dividing epithelial cancer cells (Calu-3), macrophages (RAW264.7), dendritic cells (JAWSII), as well as thymidine-induced growth-arrested cells, but not in rapidly dividing cells (A549). Among the four NLS-modified peptides, PK1 (modified with SV40 derived NLS) and PK2 (modified with reverse SV40 derived NLS) were the most consistent in improving DNA transfection; up to a 10-fold increase in gene expression was observed for PK1 and PK2 over the unmodified LAH4-L1. Additionally PK1 and PK2 were shown to enhance cellular uptake as well as nuclear entry of DNA. Overall, we show that the incorporation of SV40 derived NLS, in particular, to LAH4-L1 is a promising strategy to improve DNA delivery efficiency in slow-dividing cells and dendritic cells, with development potential for *in vivo* applications and as a DNA vaccine carrier.

INTRODUCTION

Despite the huge therapeutic potential of gene therapy including DNA vaccines, its clinical application is currently limited by the lack of a safe and effective delivery system. Non-viral vectors have the advantages of better safety profile and lower production cost compared to their viral counterparts, but their transfection efficiency is generally low.[1, 2] In order for therapeutic gene to function, plasmid DNA encoding the protein of interest must be delivered to the nucleus of the target cells to allow the transcription of DNA into the messenger RNA (mRNA), which is subsequently translated into the therapeutic proteins. DNA is a negatively charged, hydrophilic macromolecule with poor membrane permeability and is susceptible to nuclease degradation. Most non-viral vectors such as polymers, lipids and peptides are able to condense DNA to nanosize scale, either as complexes or nanoparticles, to protect the nucleic acids from degradation and facilitate cellular uptake through endocytosis.[1, 3, 4] Upon cell entry, non-viral delivery systems usually face two major intracellular barriers: (i) endosomal/lysosomal degradation; and (ii) poor nuclear entry. Therefore, the efficiency of a non-viral vector highly depends on its ability to promote endosomal/lysosomal escape and nuclear uptake. In addition, it is crucial that the DNA can be released or unloaded efficiently from the carrier so that it can be properly expressed, and the carrier itself must be non-toxic and biocompatible.

For most non-viral vectors, nuclear entry is an extremely inefficient process which limits their transfection efficiency.[1, 5, 6] Only when the cells are undergoing mitosis does the nuclear

envelope break down so that the permeability barrier to the nucleus is temporarily lost and nuclear entry can be achieved by passive diffusion.[7] Since DNA is too large to be passively transported, the only way to enter the intact nucleus of non-dividing cells is by active transport through the nuclear pore complex (NPC). This process usually requires the assistance of a nuclear localization signal (NLS), which facilitates the active transport of macromolecules into the nucleus from the cytoplasm through binding to nuclear transport proteins.[8] Different types of NLS have been reported to enhance the nuclear uptake of macromolecules. In general, NLS can be categorized as classical and non-classical. The former typically contains a series of basic, positively charged amino acid sequence while the latter does not, and they differ in binding affinity to different nuclear transport receptors.[6, 8]

The most widely studied classical NLS is the Simian Virus 40 (SV40) large T-antigen-derived NLS,[9-11] a monopartite NLS that contains a single stretch of basic amino acids. It is also the most commonly investigated NLS for the delivery of macromolecules with a well-defined nuclear import pathway. In the cytoplasm, the SV40 derived NLS is linked to the macromolecule and is recognized by the α-subunit of the importin α/β heterodimer. The β-subunit mediates the interaction with the NPC to facilitate the translocation of the trimeric complex into the nucleus.[3, 6, 12-14] Other classical NLSs include the nucleoplasmin targeting signal, which is a representative of bipartite NLSs and contains two interdependent basic amino acid clusters separated by 10 intervening neutral amino acids. Similar to SV40 derived NLS, the nucleoplasmin targeting signal also facilitates the nuclear entry of macromolecules by the importin α/β heterodimer.[6, 8, 13,

[15] In contrast, the non-classical NLSs lack the stretches of basic amino acid and they mediate the nuclear uptake through a different mechanism. The best-known example of non-classical NLS is the M9 sequence from the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1).[13, 16] The M9 sequence facilitates the transport of macromolecules across the NPC through binding to the transportin, an endogenous carrier protein distinct from the importin-mediated pathway.[16, 17] The use of NLSs to promote nuclear importation and enhance gene delivery has been reported in various non-viral gene delivery vectors including cationic polymers,[18-20] oligopeptides[21] and cell penetrating peptides.[22, 23]

Previously, our group and others have reported the use of LAH4-L1 peptide for DNA and siRNA delivery.[24-28] LAH4-L1 is a histidine-rich, cationic amphipathic peptide with pH responsive properties. At pH 7, the LAH4-L1 binds with and condenses DNA to form nano-sized complexes which enter cells through endocytosis. Once inside the acidic endosomes, LAH4-L1 becomes protonated and this causes a release of a substantial proportion of the peptide from the complex with a concomitant switch from an alpha-helical conformation to a disordered conformation in solution. The disordered LAH4-L1 is capable of binding and inserting into the endosomal membrane where it again adopts a surface aligned alpha-helix conformation capable of disordering the endosomal membrane and promoting endosomal escape of the remaining complexes.[29, 30] To further improve the DNA delivery efficiency of LAH4-L1, the strategy of incorporating NLS to the delivery peptide was explored to enhance nuclear entry. In this work, three different NLS sequences were employed: (i) the classical SV40 derived NLS; (ii) the

classical nucleoplasmin NLS; and (iii) the non-classical M9 NLS. Four NLS-modified LAH4-1 peptides, namely PK1-LAH4-L1 (PK1), PK2-LAH4-L1 (PK2), KR-LAH4-L1 (KR) and NQ-LAH4-L1 (NQ) (Table 1) were evaluated for their DNA binding affinity, transfection efficiency and the ability to promote nuclear entry. Their biological activities were tested and compared on various cell types, including A549 and Calu-3 cells which are human airway epithelial cells; RAW264.7 cells which are murine macrophage-like cells; and JAWS II cells which are murine immature dendritic cells. Furthermore, the transfection efficiency of the peptides was evaluated on A549 cells at growth-arrested state.

EXPERIMENTAL SECTION

Materials. All peptides investigated in this study (Table 1) were purchased from ChinaPeptides (Shanghai, China) at >70% purity and were used as provided. Dulbecco's modified eagle medium (DMEM), DMEM/F-12 medium, α-minimum essential medium (MEM α, nucleosides), Opti-MEM I reduced serum medium, antibiotic–antimycotic liquid, 0.25% Trypsin-EDTA, fetal bovine serum (FBS, South America origin), Lipofectamine™ 2000, Lysotracker Green DND-26 and Hoechst Stains 33258 were purchased from Invitrogen (New York or Carlsbad, USA). Recombinant murine granulocyte–macrophage colony stimulating factor (GM-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). The luciferase assay system was purchased from Promega (Madison, WI, USA). GelRed™ nucleic acid stain was purchased from Biotium (Hayward, CA, USA). Plasmid DNA (gWIZ™ Luciferase) was purchased from Aldevron (Fargo,

ND, USA). Label IT® CX-Rhodamine nucleic acid labeling kit was purchased from Mirus Bio (Madison, WI, USA). The lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI , USA). 1 × Tris-acetate EDTA (TAE) buffer was diluted from 50 × TAE buffer (242 g/L Tris base, 57.1 ml/L glacial acetic acid and 37.2 g/L EDTA, pH 8.5) using distilled water. All other reagents were obtained from Sigma Aldrich (Saint Louis, MO, USA) and of analytical grade or better.

Table 1. Sequence, molecular weight (MW), hydrophobicity, net charge and percentage charged residue of the LAH4-L1 and NLS-modified peptides. The NLS sequences are highlighted in bold and the linker group is underlined. The hydrophobicity is calculated according to the Eisenberg Consensus scale.[31]

Peptide	NLS	Sequence	MW (Da)	Average hydrophobicity	Net charge at pH 7 (% charged residue)
LAH4-L1	-	KKALLAHALHLLALLALHL AHALKKA-NH_2	2778	-0.05	+5.4 (20.8)
PK1-LAH4-L1 (PK1)	SV40	**PKKKRKV**<u>GCG</u>KKALLAHA LHLLALLALHLAHALKKA-NH_2	3861	-0.11	+10.3 (28.6)
PK2-LAH4-L1 (PK2)	'Reversed' SV40	**VKRKKKP**<u>GCG</u>KKALLAHA LHLLALLALHLAHALKKA-NH_2	3861	-0.11	+10.3 (28.6)
KR-LAH4-L1 (KR)	Nucleoplasmin	**KRPAATKKAGQAKKKK**<u>G CG</u>KKALLAHALHLLALLAL HLAHALKKA-NH_2	4717	-0.17	+13.3 (29.6)
NQ-LAH4-L1 (NQ)	M9	**NQSSNFGPMKGGNFGGRS SGPYGGGGQYFAKPRNQG GY**<u>GCG</u>KKALLAHALHLLAL LALHLAHALKKA-NH_2	6863	-0.08	+9.3 (13.9)

Gel retardation assay. For DNA binding assay, peptide/DNA complexes were prepared at 2:1, 5:1, 10:1 and 15:1 ratios (w/w) with 1 μg luciferase DNA in 25 μl TAE buffer. After leaving the complexes for 30 min at room temperature, 10 μl of samples were loaded into an agarose gel (1% w/v) stained with GelRed™. Gel electrophoresis was run in TAE buffer at 120 V for 45 min and the gel was visualized under the UV illumination. For DNA release assay, the peptide/DNA complexes were prepared at 5:1 and 10:1 ratios (w/w) as described. After 30 min, 5 μl of sodium dodecyl sulphate (SDS) solution (5 mM) was added to the complexes to allow the release of DNA. The mixtures were incubated at room temperature for 1 h. The samples were then loaded into a 1% w/v agarose gel and electrophoresis was run as described earlier. Untreated DNA and SDS treated DNA were used as controls.

Particle size and zeta potential measurement. For size measurement, peptide/DNA complexes were prepared at 10:1 ratio (w/w) with 4 μg luciferase DNA in 100 μl ultrapure water. After leaving the mixture for 30 min at room temperature, the hydrodynamic diameter and the polydispersity index of the complexes were measured by photon correlation spectroscopy (Delsa^{TM}-Nano C, Beckman Coulter, CA, USA). For zeta potential measurement, the peptide/DNA complexes were prepared at 10:1 ratio (w/w) with 20 μg luciferase DNA in 500 μl of 2% PBS. The mixture was then incubated at room temperature for 30 min and the zeta potential was measured in a flow cell using electrophoretic light scattering (Delsa^{TM}-Nano C, Beckman Coulter, CA, USA).

Morphology study. The peptide/DNA complexes were prepared at 10:1 ratio (w/w) with 2 μg luciferase DNA in 200 μl ultrapure water and the complexes were left at room temperature for at least 30 min. A drop of sample (around 30 μl) was placed onto a discharged copper grid coated with carbon-formvar for 30 s, followed by staining with 4% (w/v) uranyl acetate for 1 min. The grid was then washed with distilled water once. Excessive solution was carefully removed by filter paper prior to air-drying. The samples were analyzed under the transmission electron microscope (TEM) (Philips CM100 TEM, Philips Electron Optics, Eindhoven, Netherlands) at a voltage of 100 kV. Micrographs were taken using a digital camera (Olympus, TENGRA 2.3K × 2.3K bottom mounted camera system with iTEM acquisition software).

Cell culture. A549, Calu-3, RAW 264.7 and JAWSII cells were obtained from American Type Culture Collection (Manassas, VA, USA). Calu-3 cells were grown in DMEM/F-12 medium supplemented with 10% v/v FBS and were subcultured once a week; A549 and RAW264.7 cells were grown in DMEM supplemented with 10% FBS and were subcultured every three days; JAWSII cells were cultured in α-minimum essential medium supplemented with 20% v/v FBS and 5 ng/ml recombinant mouse granulocyte–macrophage colony stimulating factor (GM-CSF) and were subcultured once a week. All the media contained 1% v/v antibiotic/antimycotic liquid and all the cells were maintained at 37°C, 5% CO_2.

DNA transfection. A549, Calu-3, RAW264.7 and JAWSII cells were transfected with peptide/DNA complexes at 1 μg luciferase plasmid per well in 24-well plates. Lipofectamine™ 2000 was used as control. The complexes were prepared at 5:1, 10:1 and 15:1 ratios (w/w) in Opti-MEM I reduced serum medium. The cells were transfected when they reached 70-80% confluency. After 5 h of incubation with the complexes at 37 °C, the transfection media were removed and replaced with serum supplemented cell culture media. At 24 h post-transfection, the cells were rinsed and lysed in 100 μl reporter lysis buffer. The luciferase expression was detected using the luciferase assay system according to the manufacturer's protocol. The results were expressed as relative light unit (RLU) per mg of total protein. For transfection with growth-arrested cells, the standard double thymidine block method was employed as previously reported.[32] A549 cells were seeded at a density of 5×10^4 cells per well in 24-well plate for overnight. The cell culture medium was then replaced with one containing thymidine (2mM). After 19 h of incubation, the cells were washed and incubated with thymidine-free culture medium. After 9 h, thymidine was added to the culture medium and the cells were incubated for a further 17 h. The cells were washed again. Peptide/DNA complexes prepared in Opti-MEM I reduced serum medium containing thymidine were added to the cells. After 5 h of incubation, the transfection medium was removed and replaced with fresh culture medium. The luciferase expression was examined at 24 h post-transfection as described above.

Cellular uptake assay. DNA was fluorescently labelled with rhodamine using the Label IT® CX-Rhodamine nucleic acid labeling kit, which is commonly used for intracellular trafficking

studies of gene delivery system,[18, 33] according to the manufacturer's protocol. Calu-3, RAW264.7 and JAWSII cells were seeded onto 6-well culture plates at a seeding density of 4 × 10^5 cells per well. After 24 to 48 h, the cells were washed with PBS and transfected with peptide/DNA complexes (prepared at 10:1 ratio w/w) containing 2 μg of labelled DNA per well in Opti-MEM I reduced serum medium. After 5 h of incubation, the transfection medium was removed and the cells were washed with warm PBS. The cells were then detached from the plates using 0.25% w/v Trypsin-EDTA or 0.02% w/v EDTA-EGTA in PBS. To quench the extracellular fluorescence signal, trypan blue solution (0.04% w/v) prepared in PBS was added to the cells for 2 min. The cells were then centrifuged and washed with PBS thrice. The cells were re-suspended in 500 μl PBS and sieved with a sterile 40 μm cell strainer (BD Biosciences, CA, USA). The fluorescence intensity of the transfected cells was analyzed by flow cytometer (BD LSR Fortessa Analyzer, BD Bioscience, USA). The cells were gated electronically according to the non-transfected cell forward-scatter (FSC) and side-scatter (SSC) properties to induce the main population of cells and to exclude dead cells. At least 10,000 events were analyzed for each sample. The results were expressed as percentage of cells displayed positive fluorescence signal compared to the untreated cells. The mean fluorescence intensity of the treated cells was also calculated and compared.

Nuclear uptake assay. The nuclear isolation and DNA quantification method was adopted from previous studies with modification.[21, 34, 35] RAW264.7 and JAWSII cells were seeded onto 6-well plates at a seeding density of 5 × 10^5 cells per well for overnight. The cells were washed with

PBS and transfected with peptide/DNA complexes (prepared at 10:1 ratio w/w) containing 2 μg of rhodamine labelled DNA per well in Opti-MEM I reduced serum medium. After 5 h, the transfection medium was removed and replaced with serum-supplemented culture media. The cells were washed with PBS and detached from the plates at 24 h post-transfection using EDTA-EGTA (0.02% w/v). To collect the cell nuclei, the cells were incubated in hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4) on ice for 10 min. 500 μl lysis buffer (1% v/v Nonidet P-40, 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, pH7.4) were added to the cells. The mixtures were vortexed for 5 to 10 s and incubated for 2 min at room temperature to dissolve the cell membranes. The lysis of cell membrane and the maintenance of nuclear integrity were monitored under a microscope. After centrifugation at 1,400 × g for 5 min, the supernatant (cytosolic fraction) was removed and the pellets (nuclear fraction) were washed with PBS and re-suspended in 500 μl PBS for nuclei counting and fluorescence detection. The number of nuclei was counted using a hemocytometer after staining with 0.5% Trypan Blue solution in PBS (v/v). The fluorescent intensity of the labelled DNA in the nuclei was analyzed by a fluorescent microplate reader (CLARIOstar®, BMG Labtech, Germany). The amount of labelled DNA in the nuclei was calculated using a standard curve and the results were shown as the amount of DNA per nucleus.

Confocal live cell imaging. Calu-3 cells were seeded on a 35 mm Mattek glass bottom culture dish (Mattek Corp. Ashland, MA) with 4×10^5 cells per well for overnight. Peptide/DNA complexes (10:1 ratio w/w) were prepared using rhodamine labelled DNA in 1 ml of Opti-MEM

I reduced serum medium and were added to the cells after 30 min of incubation. The complexes were removed and replaced with serum-supplemented culture medium after 5 h of incubation. The cells were imaged at 24 h post-transfection. Prior to imaging, the nuclei of the cells were stained with Hoechst (5 μg/ml) for 30 min. The lysosomes were labelled with Lysotracker (50 nM) 2 min before imaging. During live cell imaging, the cells were incubated at 37 °C and 5% CO_2 in a heated stage-top incubator. The confocal laser scanning microscope (Zeiss LSM 780 inverted microscope, Jena, Germany) with diode laser (405 nm), argon laser (488 nm) and diode-pumped solid state (DPSS) laser (561 nm) were used to visualize the cells. The microscope was observed with 40 × 1.4 oil DIC objective lens. The images were analyzed by Zeiss LSM Image Browser (Version 8.1.0.484, Jena, Germany).

Cytotoxicity study. Calu-3 and JAWSII cells were seeded in 96-well plates at a cell density of 4 × 10^4 cells per well for 48 h. Peptide/DNA complexes (prepared at 10:1 ratio w/w) containing 0.5 μg luciferase plasmid per well were diluted in 200 μl of culture medium. Triton X-100 (1% v/v) treated cells and untreated cells were used as positive and negative controls respectively under the same condition. At 5 h post-transfection, 100 μl cell supernatant from each well was transferred to a new 96-well plate and the LDH activity was determined using LDH cytotoxicity assay kit according to the manufacturer's protocol. The relative LDH release was calculated by the percentage of LDH release in the sample over control, with 1% Triton X-100 treated cells were set as 100% LDH release and the untreated cells were set as 0% LDH release.

RESULTS

DNA affinity. The peptides were evaluated for their affinity to DNA. In the DNA binding study (Fig. 1), the shift or disappearance of DNA band in the gel indicated binding occurred between DNA and peptides. For LAH4-L1 and NQ, DNA binding was observed at 5:1 ratio (w/w) onwards, whereas for PK1, PK2 and KR, DNA binding was detected at a lower ratio of 2:1 (w/w), suggesting that PK1, PK2 and KR had higher DNA affinity than LAH4-L1 and NQ. In the DNA release study, the anionic surfactant SDS was used to dissociate the peptide/DNA complexes by competing with DNA for the cationic peptides (Fig. 2). Since all the peptides could bind to DNA at ratio 5:1 (w/w) or above, the recovery of DNA band in the gel suggested that the DNA could be released from the complexes by SDS. For LAH4-L1, PK1 and PK2, the release of DNA was observed at 5:1 and partially at 10:1 ratios (w/w), whereas for KR, the partial release of DNA was only observed at 5:1 ratio (w/w). The binding between KR and DNA at ratio 10:1 (w/w) was probably too strong to be released by the SDS, suggesting that KR had the highest DNA affinity. Overall, the DNA affinity of the peptides was in the descending order of KR > PK1 and PK2 > LAH4-L1 and NQ.

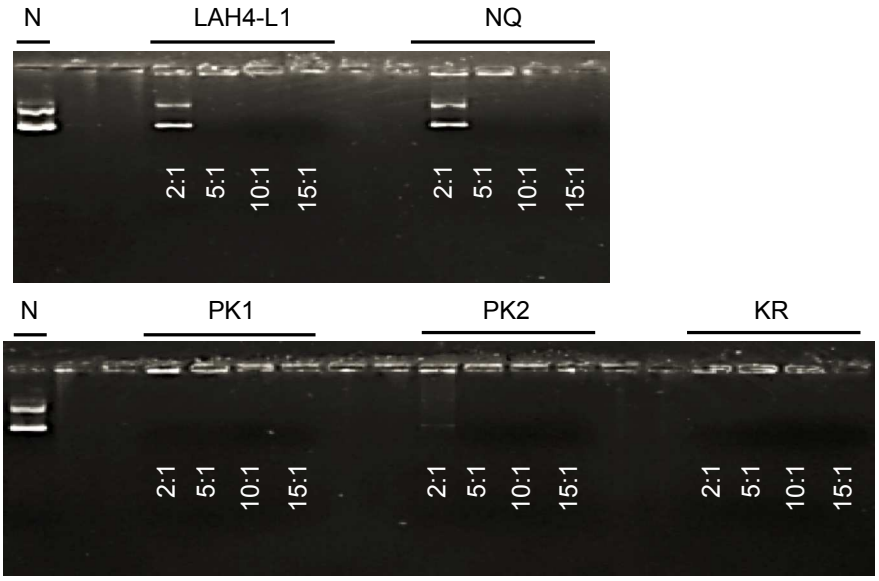


Figure 1. Agarose gel retardation assay of DNA binding. Peptide/DNA complexes were prepared at 2:1, 5:1, 10:1 and 15:1 ratios (w/w). Naked DNA (N) were used as controls. At 30 min after complex formation, the samples were loaded into the agarose gel (1% w/v) stained with GelRed™. Electrophoresis was carried out at 120 V for 45 min and the gel was visualized under the UV illumination.

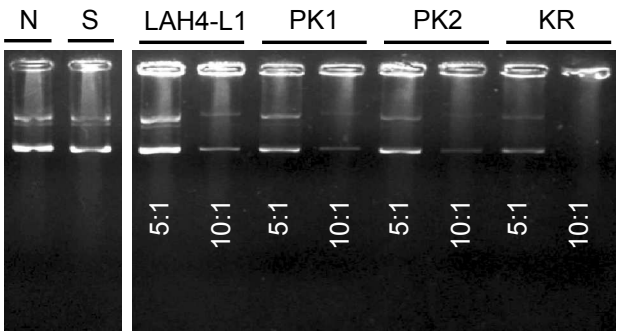


Figure 2. Agarose gel retardation assay of DNA release. Peptide/DNA complexes were prepared at 5:1 and 10:1 ratios (w/w). At 30 min after complex formation, SDS (5 mM) was added to allow dissociation of complexes. After 1 h, the mixtures were loaded into an agarose gel (1% w/v) stained with GelRed™. Naked DNA (N) and SDS treated DNA (S) were used as controls. Electrophoresis was carried out at 120 V for 45 min and the gel was visualized under the UV illumination.

Particle size and zeta potential analysis. The particle size distribution and the zeta potential of peptide/DNA complexes prepared at 10:1 ratio (w/w) were determined by photon correlation spectroscopy and electrophoretic light scattering, respectively (Table 2). All the peptides formed nano-sized complexes with DNA ranging from 130 nm to around 350 nm. The polydispersity index of the complexes was similar, ranging between 0.19 and 0.27. Among these complexes, those formed with PK1, PK2 and KR were below 200 nm, and they were smaller than the complexes formed with LAH4-L1, which were over 200 nm. NQ formed the largest DNA complexes which were over 300 nm. For zeta potential measurement, all the complexes formed were positively charged. Complexes formed with KR had the highest zeta potential of around +24 mV, followed by PK1 and PK2 which had the zeta potential between +10 to +13 mV. The complexes formed with LAH4-L1 were only slightly positive charged with a zeta potential value of +2 mV.

Table 2. Hydrodynamic diameter, polydispersity index and zeta potential of peptide/DNA complexes prepared at 10:1 ratio (w/w) measured by photon correlation spectroscopy and electrophoretic light scattering (n=4). (N.A. data not available due to insufficient material)

Peptides	Diameter ± SD (nm)	Polydispersity index ± SD	Zeta Potential ± SD (mV)
LAH4-L1	226.7 ± 31.7	0.24 ± 0.05	+2.2 ± 0.9
PK1	164.9 ± 1.3	0.27 ± 0.02	+10.1 ± 2.1
PK2	130.1 ± 1.1	0.26 ± 0.01	+13.3 ± 1.6
KR	184.6 ± 6.8	0.27 ± 0.02	+24.2 ± 0.9
NQ	354.5 ± 81.5	0.20 ±0.01	N.A.

Morphology study. The morphology of peptide/DNA complexes was examined by TEM (Fig. 3). As shown in the microscope images, all the peptides investigated (LAH4-L1, PK1, PK2 and KR) were able to form nano-sized, compact but irregularly shaped complexes with DNA, and the morphology of the complexes formed with different peptides were all very similar.

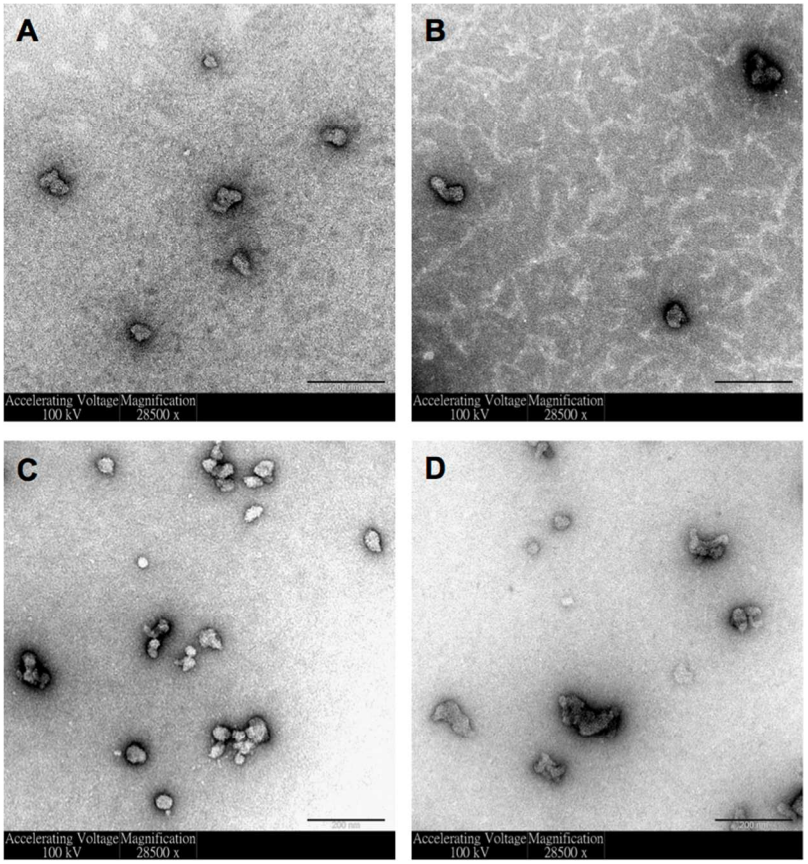


Figure 3. TEM images of peptide/DNA complexes - LAH4-L1/DNA complexes (A); PK1/DNA complexes (B); PK2/DNA complexes (C); KR/DNA complexes (D). All the complexes were prepared at 10:1 ratio (w/w) in ultrapure water and stained with 4% (w/v) uranyl acetate. Scale bar = 200 nm

Luciferase DNA transfection efficiency. DNA delivery efficiency was examined by luciferase activity on four different cell lines after transfected with peptide/DNA complexes prepared at 5:1 to 15:1 ratios (w/w) (Fig. 4). Comparing to LAH4-L1, none of the NLS-modified peptides showed significant improvement of transfection on A549 cells. PK1 and PK2 were able to improve DNA transfection efficiency significantly over the LAH4-L1 ($p < 0.05$) on other three cell lines. On Calu-3 cells, both peptides showed approximately 9-fold increase in transfection efficiency over LAH4-L1 at 10:1 ratio (w/w). On RAW264.7 cells, significant improvement was observed at all three ratios, with the largest increase by around 6-fold at 5:1 ratio (w/w) for both PK1 and PK2. On JAWSII cells, there was up to 5.5- to 6.0-fold increase in transfection efficiency over LAH4-L1 for PK1 and PK2 at 10:1 ratio (w/w). KR was able to improve DNA transfection efficiency of LAH4-L1 significantly ($p < 0.05$) on JAWSII cells (at 10:1 and 15:1 ratios w/w) only, with up to 9.4-fold of increase in gene expression observed at 10:1 ratio (w/w). KR also mediated transfection on RAW264.7 cells about one-fold higher than LAH4-L1, but the change was not statistically significant. NQ failed to improve the transfection over LAH4-L1 on all four tested cell lines. In addition, PK1, PK2 and KR (at 10:1 and 15:1 ratios w/w) also performed significantly better than Lipofectamine™ 2000 ($p < 0.05$) on JAWSII cells. For transfection on growth-arrested cells (Fig. 5), A549 cells were kept at non-dividing state by double thymidine block. A significant improvement in transfection was observed with PK1 peptide ($p < 0.01$) and PK2 ($p < 0.001$), with around 1.4-fold and 1.7-fold of increase in gene expression respectively compared to LAH4-L1. However, KR failed to mediate DNA transfection when A549 cells were non-dividing.

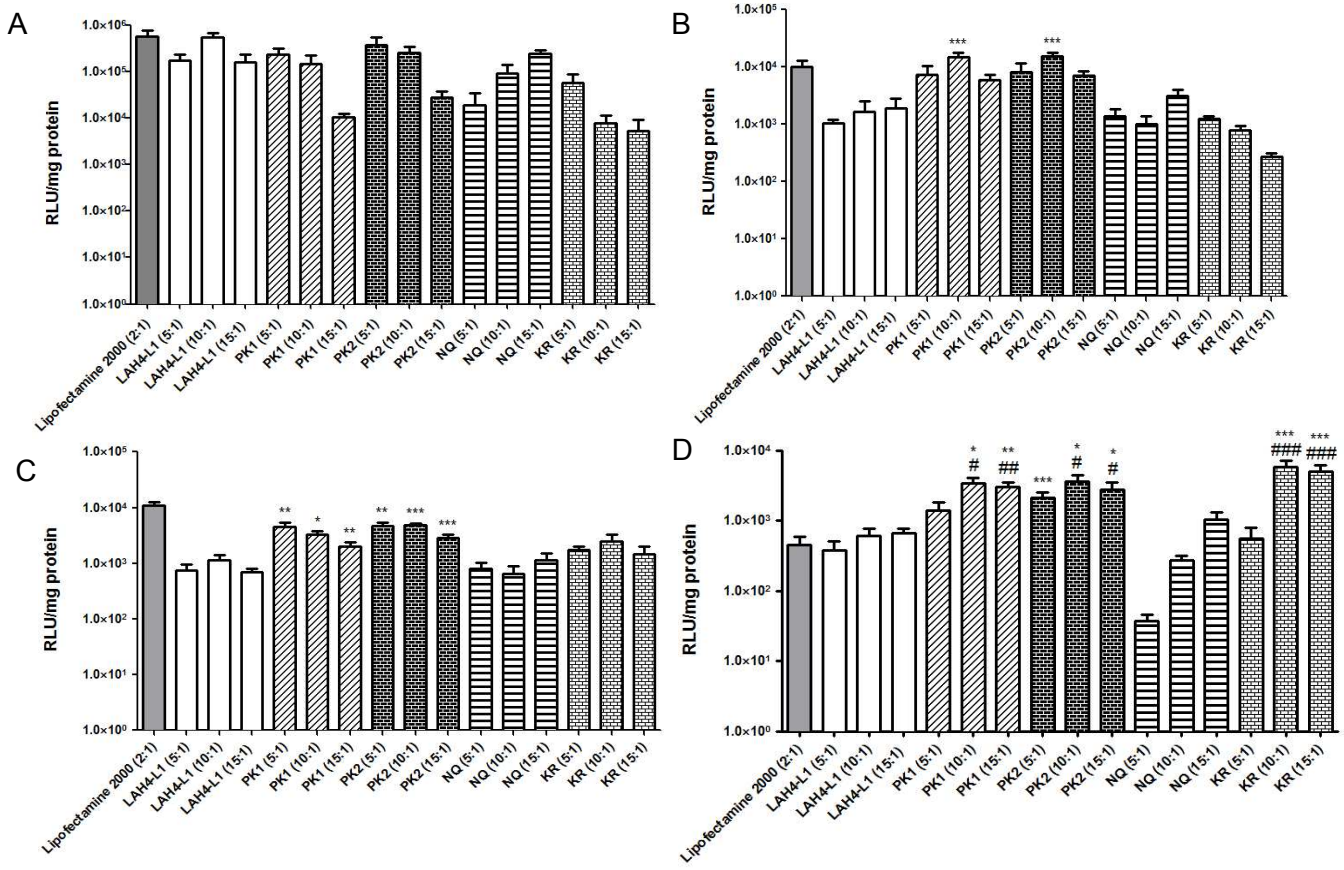


Figure 4. Luciferase DNA transfection study. Cells were transfected with peptide/DNA complexes prepared at 5:1, 10:1 and 15:1 ratios (w/w), with 1 μg DNA per well in 24-well plates. Luciferase expression was measured at 24 h post-transfection. Lipofectamine™ 2000 (lipid/DNA 2:1 ratio w/w) was used as control. Transfection was carried out on A549 cells (A); Calu-3 cells (B); RAW264.7 cells (C); and JAWSII cells (D). The data were analyzed by one-way ANOVA followed by Bonferroni *post-hoc* test. * ($p < 0.05$); ** ($p < 0.01$); *** ($p<0.001$) compared with LAH4-L1 at the same ratio (w/w); $^{\#}$ ($p < 0.05$); $^{\#\#}$ ($p < 0.01$); $^{\#\#\#}$ ($p<0.001$) compared with Lipofectamine 2000. Values are the mean ± SEM (n=3-4).

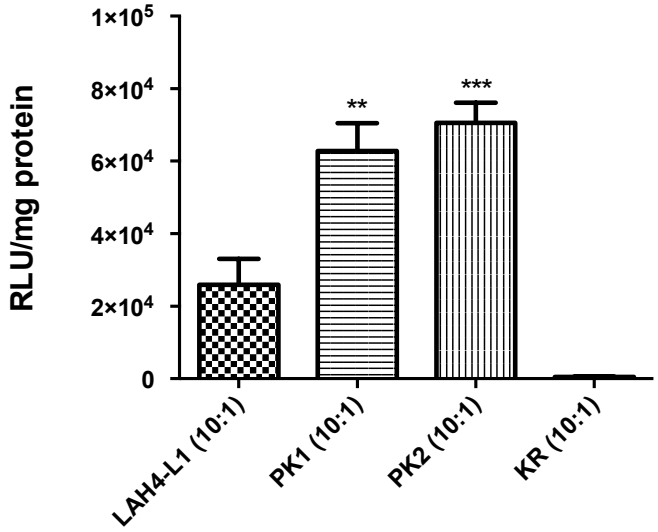
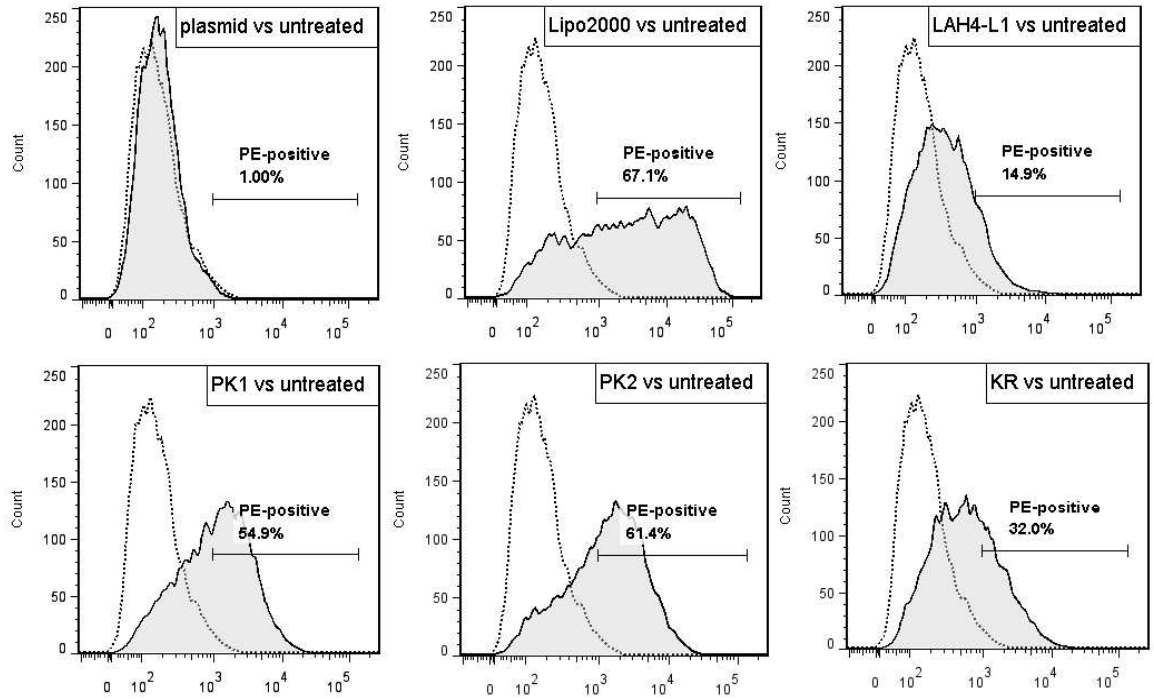


Figure 5. Luciferase DNA transfection study on growth-arrested A549 cells. The cells were kept in growth-arrested state (non-dividing) using double thymidine block. Cells were transfected with peptide/DNA complexes prepared at 10:1 ratio (w/w), with 1 μg DNA per well in 24-well plates. Expression of luciferase was measured at 24 h post-transfection. The data were analyzed by one-way ANOVA followed by Bonferroni *post-hoc* test. ** ($p < 0.01$); ***($p < 0.001$) compared with LAH4-L1. Values are the mean ± SEM (n=4).

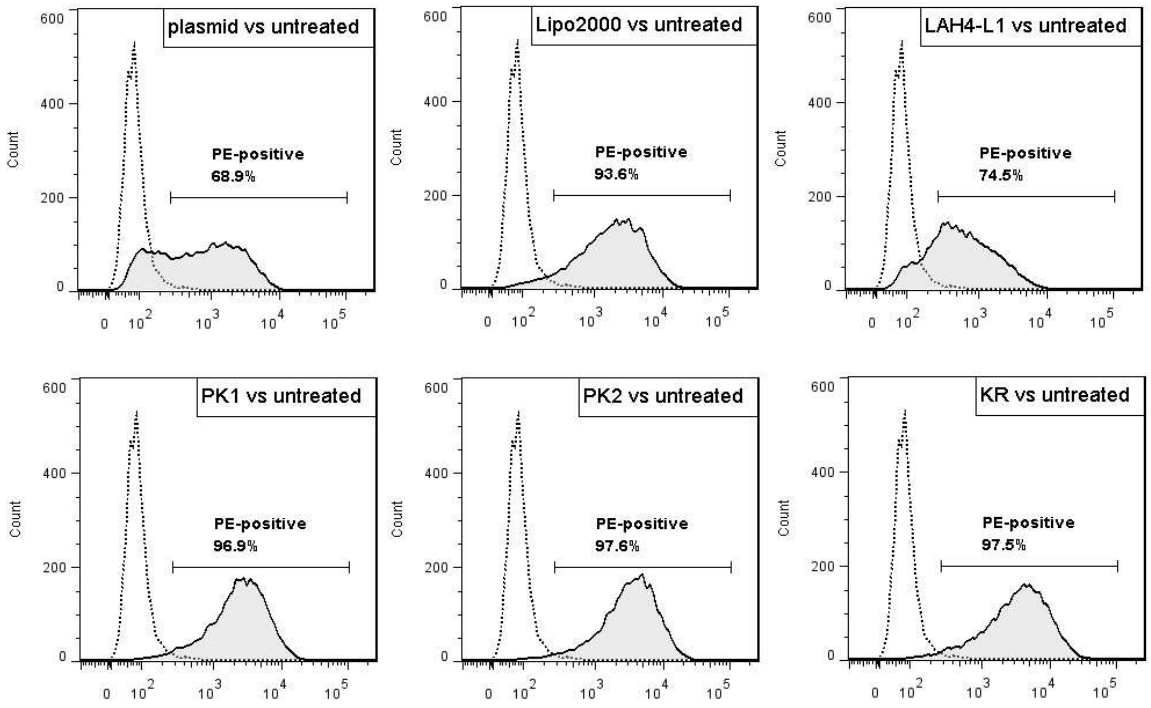
Cellular uptake study. The cellular uptake of fluorescently labelled DNA was measured by flow cytometry (Fig. 6). A549 cells were not used because none of the NLS-modified peptides showed significant improved transfection on this cell line when the cells are dividing. NQ, which failed to show any increase in transfection over LAH4-L1 in any of the cell lines, was also excluded. The peptide/DNA complexes were prepared at 10:1 ratio w/w. The histogram analysis (Fig. 6 A-C) showed the fluorescent-positive cell populations in percentage (shaded) as

compared to the fluorescent-negative untreated control (unshaded). PK1, PK2 and KR all showed a higher percentage of fluorescent-positive cells than LAH4-L1 in all three cell lines. A similar trend was observed when compared the mean fluorescence intensity of the treated cells (Fig. 6 D-F). The three NLS-modified peptides showed significant increase ($p < 0.05$) in fluorescence intensity over Lipofectamine 2000 and LAH4-L1 on JAWSII cells, but not on the other two cell lines.

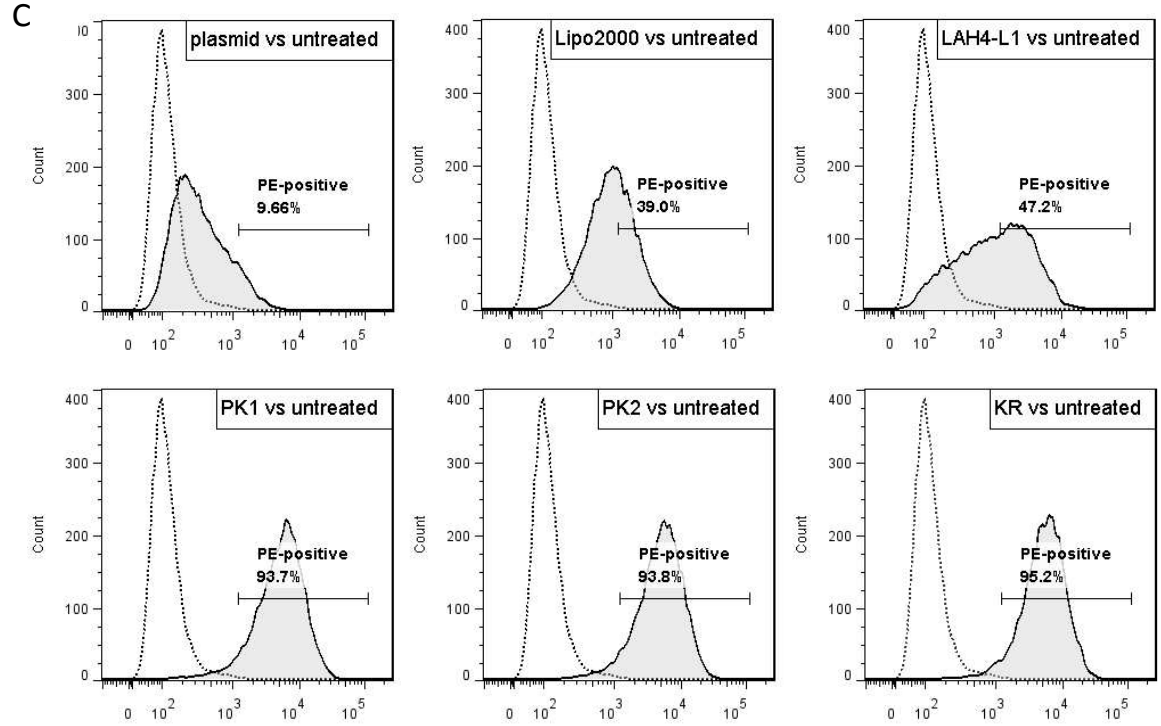
A



B


plasmid vs untreated
PE-positive
68.9%
Lipo2000 vs untreated
PE-positive
93.6%
LAH4-L1 vs untreated
PE-positive
74.5%
PK1 vs untreated
PE-positive
96.9%
PK2 vs untreated
PE-positive
97.6%
KR vs untreated
PE-positive
97.5%
Count

C


plasmid vs untreated
PE-positive
9.66%
Lipo2000 vs untreated
PE-positive
39.0%
LAH4-L1 vs untreated
PE-positive
47.2%
PK1 vs untreated
PE-positive
93.7%
PK2 vs untreated
PE-positive
93.8%
KR vs untreated
PE-positive
95.2%
Count

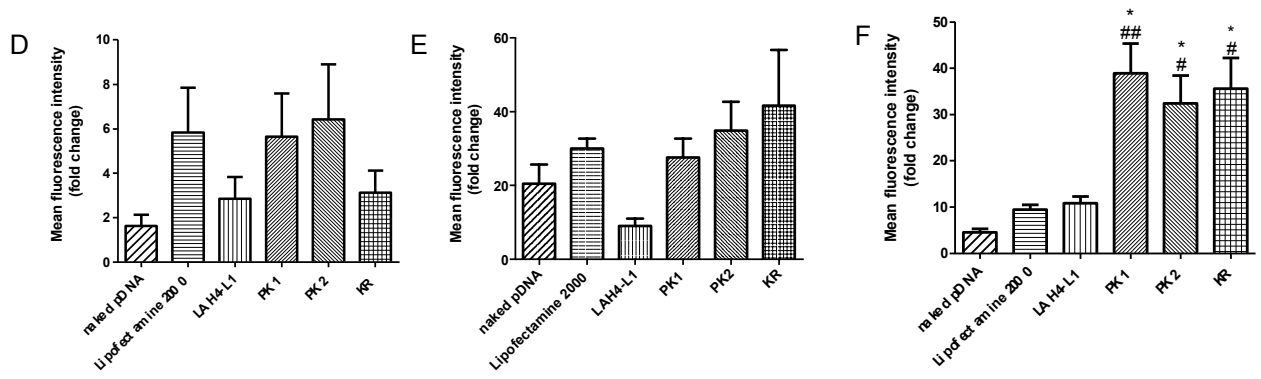


Figure 6. Cellular uptake study of fluorescently labelled DNA by flow cytometry. Cells were transfected with peptide/DNA complexes prepared at 10:1 ratio (w/w) at 2 μg per well in 6-well plates. Naked DNA and Lipofectamine 2000 (lipid/DNA at 2:1 ratio w/w) were used as controls. Transfection was carried out on Calu-3 cells (A & D); RAW264.7 cells (B & E); and JAWSII cells (C & F). The mean fluorescence intensity of the cells was measured at 5 h post-transfection. The histograms of fluorescence intensity and the percentage of cells that displayed positive fluorescence signal (shaded) compared to untreated cells (unshaded) were shown (A-C). The fluorescence intensity was also expressed as fold change compared to the untreated cells (D-F). The data was analysed by one-way ANOVA followed by Bonferroni *post-hoc* test. * ($p < 0.05$) compared with LAH4-L1 at the same ratio (w/w); $^{\#}$ ($p < 0.05$); $^{\#\#}$ ($p < 0.01$) compared with Lipofectamine 2000. Values are the mean ± SEM (n=3-4).

Nuclear import study. The nuclear uptake of fluorescently labelled DNA was quantified in the isolated nuclei of the cells after they were transfected using LAH4-L1, PK1 or PK2 (Fig. 7). Due to the highly aggregating nature of Calu-3 cells[36] which often led to incomplete cell lysis during the nuclei isolation process, this study was carried out on RAW264.7 and JAWSII cells only (the live cell confocal imaging was used to visualize the nuclear localization in Calu-3 cells). Similar to the cellular uptake study, the peptide/DNA complexes were prepared at 10:1 ratio (w/w). Both

PK1 and PK2 were able to enhance the transportation of DNA into the nucleus significantly ($p < 0.05$) on both RAW264.7 and JAWSII cells when compared to LAH4-L1. In addition, PK1 and PK2 also mediated higher nuclear uptake of DNA over Lipofectamine™ 2000.

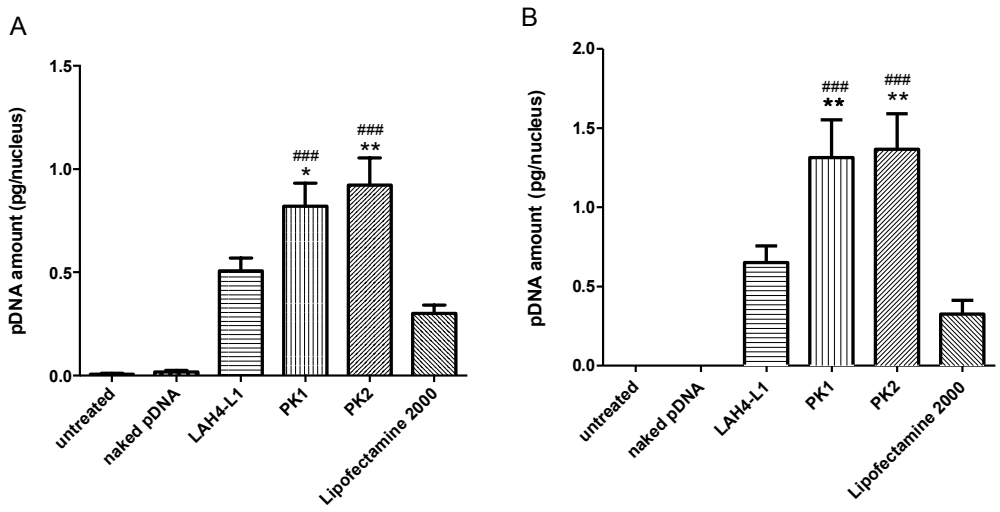


Figure 7. Nuclear uptake study of DNA. Fluorescently labelled DNA was used to prepared peptide/DNA complexes at 10:1 ratio (w/w) at 2 μg per well in 6-well plates. Naked DNA and Lipofectamine™ 2000 (lipid/DNA 2:1 ratio w/w) were used as controls. Transfection was carried out on RAW264.7 cells (A) and JAWSII cells (B). At 24 h post-transfection, cell nuclei were isolated and the amount of labelled DNA was quantified by measuring the fluorescence level compared to a standard curve. The data was compared and analyzed by one-way ANOVA followed by Bonferroni *post-hoc* test. *($p<0.05$) and **($p<0.01$) compared to cells transfected with LAH4-L1; ### ($p<0.001$) compared to cells transfected with Lipofectamine 2000. Values are the mean ± SEM (n=4).

Live cell confocal imaging. The localization of exogenous DNA delivered by LAH4-L1 and PK2 was compared on Calu-3 cells (Fig. 8). The DNA was fluorescently labelled with rhodamine (red), the nuclei were stained with Hoechst 33258 (blue), and the lysosomes were

labelled with LysoTracker (green). The cells were imaged at 24 h post-transfection by live cell imaging. The nuclear localization of exogenous DNA was observed in cells transfected with PK2, as indicated by the arrows in the image (Fig. 8H), but not in LAH4-L1. Moreover, the co-localization between DNA and lysosomes (as seen in yellow) was observed with cells transfected with LAH4-L1 but not with those transfected with PK2.

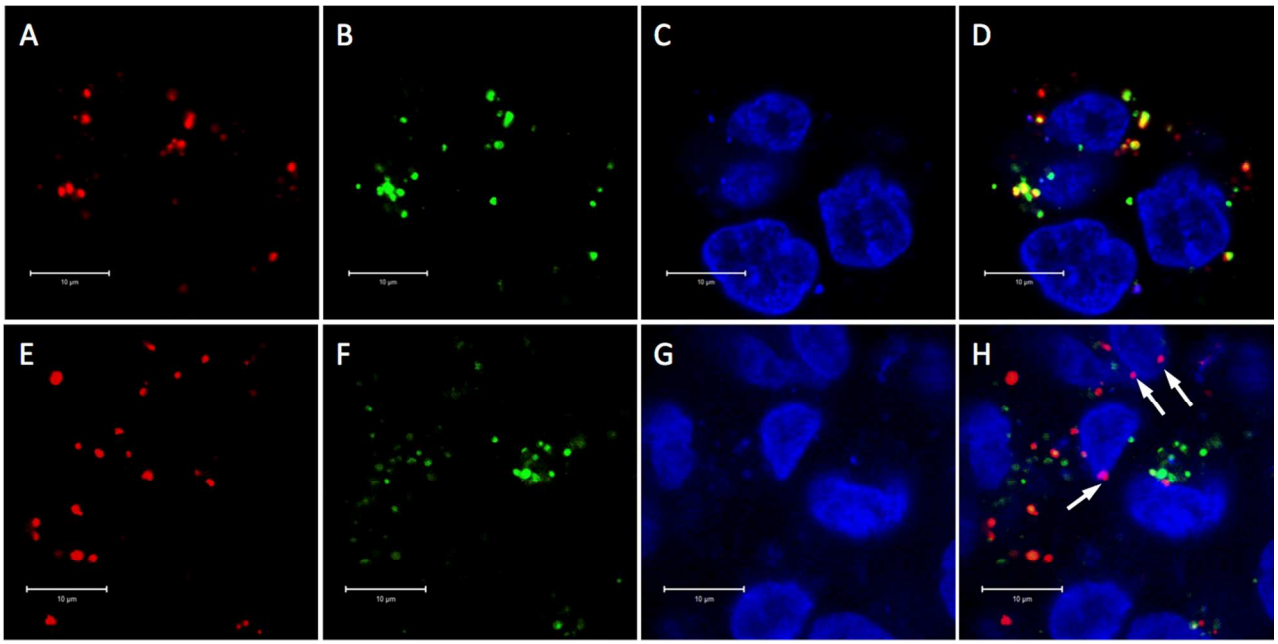


Figure 8. Localization of DNA using confocal live cell imaging. Calu-3 cells were transfected with DNA using LAH4-L1 (A-D) or PK2 (E-H), and the peptide/DNA complexes were prepared at 10:1 ratio (w/w). The images were taken at 24 h post-transfection. Luciferase DNA (red) was labelled with rhodamine (A & E); LysoTracker (green) was used to label the lysosomes (B & F); the nuclei (blue) were stained with Hoechst (C & G) and the overlaid images were shown (D & H). The white arrows indicate the co-localization of DNA and nucleus. Scale bar = 10 μm.

Cytotoxicity study. The cytotoxicity of peptide/DNA complexes on Calu-3 and JAWSII cells was evaluated by LDH assay (Fig. 9). No sign of toxicity was observed in Calu-3 cells after exposure to the complexes, suggesting that the peptides were not toxic to epithelial cells at concentration used for transfection. For JAWSII cells, there was no sign of toxicity in cells treated of NLS-modified peptide/DNA complexes, and just below 20% of LDH release was observed in cells treated with LAH4-L1/DNA complexes. The results suggested that LAH4-L1 appeared to be more cytotoxic than the NLS-modified peptides in dendritic cells.

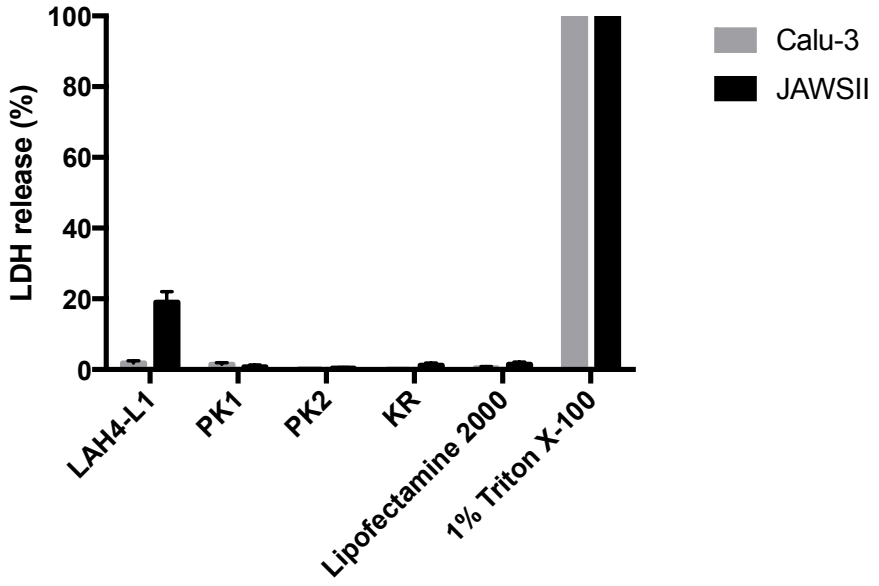


Figure 9. Cytotoxicity study using lactate dehydrogenase (LDH) release assay on Calu-3 and JAWSII cells. Peptide/DNA complexes were prepared at 10:1 w/w ratio at 0.5 μg per well in 96-well plates. Triton X-100 (1% v/v) were used as positive controls. LDH assay was carried out at 5 h post-transfection. Results were presented as percentage of LDH release relative to the controls (Triton X-100 treated cells were set as 100% LDH release and the untreated cells were set as 0% LDH release). The bars were shown as mean ± SEM (n=3-4).

DISCUSSION

Nuclear entry is a major hurdle to effective DNA delivery. One of the strategies to overcome this barrier is by incorporation of NLS to the delivery system. It was previously demonstrated that LAH4-L1, a pH responsive peptide, was able to mediate effective transfection of nucleic acids in mammalian cells.[24] In this study, LAH4-L1 was modified with NLS with the attempt to further improve the transfection efficiency by enhancing the nuclear uptake of DNA. The advantage of attaching the NLS to the carrier instead of the DNA directly is to avoid any possible interference with the transcription domain of the DNA,[37, 38] as well as the more flexible application to different kinds of genes. Here, three NLSs were investigated, including two classical NLSs (SV40 derived NLS and nucleoplasmin targeting signal) and one non-classical NLS (M9 sequence). In addition, the reverse SV40 derived NLS was also examined. A simple construct was employed, with NLS attached to the N terminal of LAH4-L1 and a short glycine-rich linker (GCG) in between to separate the two domains.

LAH4-L1 carries a net positive charge, which allows it to form complexes with the negatively charged DNA through electrostatic interaction. The classical NLSs typically contain series of positively charged amino acid residues, therefore the addition of this type of NLS leads to an increase of overall charge of the peptide. Due to the difference in charge density between the four NLS-modified peptides, their affinity for DNA would be different. According to the gel retardation assay, KR displayed the strongest affinity for DNA, followed by PK1 and PK2,

whereas LAH4-L1 and NQ had the weakest DNA affinity. As expected, the result reflected that the peptides with higher charge density (KR, PK1 and PK2 – all contained classical NLS) exhibited a higher DNA binding affinity than those with lower charge density (the unmodified LAH4-L1 and the non-classical NLS modified NQ). This is also consistent with the zeta potential measurement that the DNA complexes formed with peptides containing classical NLS were more positively charged than those formed with LAH4-L1, with KR complexes exhibited the highest positive potential. Moreover, the particle size measurement showed that peptides with higher DNA binding affinity or at higher peptide to DNA weight ratio (Table S1) tended to form DNA complexes with smaller size.

A good balance between DNA binding and release must be maintained in order to allow the formation of stable peptide/DNA complexes for effective cellular uptake, as well as the dissociation of the complexes inside the cells for DNA transcription to occur. Since effective DNA binding was achieved at peptide to DNA ratio of 5:1 (w/w) for all peptides, the subsequent transfection study was carried out using complexes prepared at this ratio and above to identify the optimal ratio for each peptide. Across the four tested cell lines, for LAH4-L1, the highest DNA transfection was achieved at ratio 10:1 to 15:1 (w/w). For peptides containing classical NLS (PK1, PK2 and KR), the highest transfection was obtained at a lower ratio of 5:1 to 10:1 (w/w). For NQ, the highest transfection was observed at ratio 15:1 (w/w). This trend closely correlated with the DNA binding affinity of the peptides - the stronger the DNA affinity of the

peptides, the smaller the amount of peptide (i.e. lower peptide to DNA ratio) required for effective transfection.

Both A549 and Calu-3 cells are human epithelial cancer cell lines that are commonly used for evaluating the performance of DNA delivery agents through intranasal or pulmonary administration. These routes of administration offer potential advantage of avoiding interaction with serum and the rapid nuclease degradation that occurs in the bloodstream.[39] Compared to LAH4-L1, none of the NLS-containing peptides showed significant improvement in gene expression on A549 cells. This was not surprising as A549 cells are rapidly dividing cells, nuclear uptake of DNA could be achieved easily when the nuclear membrane is disassembled during cell mitosis. If the nuclear uptake were not a limiting factor, incorporation of NLS to the peptide would not be expected to cause any significant increase in gene expression. Our finding is consistent with other studies that report that the contribution of NLS in gene delivery was unnecessary or less remarkable in dividing cells.[18, 40] To examine the role of cell division on DNA transfection, A549 cells were growth-arrested by a standard double thymidine blocking procedure.[32, 41] Under such treatment condition, the cells were synchronized and kept at non-dividing state, and the transfection results showed that PK1 and PK2 were more efficient in mediating DNA transfection than LAH4-L1. Furthermore, PK1 and PK2 were able to significantly enhance transfection over the LAH4-L1 on Calu-3 cells. Since Calu-3 cells have a much slower cell division rate than A549 cells, the nuclear uptake is inefficient and becomes a crucial barrier that impedes the import of DNA into the nucleus. PK1 and PK2 contained the

SV40 NLS and 'reversed' SV40 NLS respectively, suggesting that SV40 NLS has a role in facilitating DNA transfection, possibly by promoting nuclear import in part. However, such an improvement was not observed in KR and NQ.

DNA vaccine is an important application of gene therapy, requiring the expression of DNA encoding antigen in antigen presenting cells (APCs) to induce sufficient immune responses. The potential of the peptides as DNA vaccine carrier was explored. Two APCs were included in this transfection study, RAW264.7 cells (macrophages) and JAWSII cells (dendritic cells). Both PK1 and PK2 outperformed LAH4-L1 on the two cell lines, whereas KR only showed significantly better transfection than LAH4-L1 on JAWSII cells. Moreover, PK1, PK2 and KR peptides all performed significantly better than Lipofectamine 2000 on JAWSII cells. Combined peptide systems containing LAH4-L1 and NLS-modified peptides at different ratios were also examined. The rationale of studying the combined peptide system is to optimize the ratio of NLS to DNA without changing the total amount of peptides. DNA complexes formed with the combined peptide system were around 200 nm or below (Table S2), and the transfection study on JAWSII cells showed that all NLS-modified peptides, PK1, PK2 and KR, performed better when used alone than in combination with LAH4-L1 (Fig. S1). Dendritic cells are hard to be transfected by non-viral vectors. Apart from their slowly dividing property, they are professional APCs that strictly control the trafficking of molecules across their cell membranes and difficult to transfer genes into the nucleus. Although effective gene transfer was observed using viral vectors, the viruses may influence the functions and immune response of the cells which are not desirable.[42,

[43] To investigate the safety profile of these peptides and hence their suitability for clinical development, cytotoxicity study was performed by measuring the LDH leakage of cells treated with the peptide/DNA complexes. LDH is a soluble cytoplasmic enzyme which is released into the surrounding medium upon membrane damage or cell lysis. Since LAH4-L1 peptide is membrane active at low pH (a mechanism to promote endosomal escape), it is crucial to examine the membrane integrity of cells after transfected with LAH4-L1 and its NLS-modified analogues. There was no sign of cytotoxicity for PK1, PK2 and KR peptides, indicating that they have the potential for further investigation on other antigen-presenting cells as DNA vaccine carriers.

NQ which contains M9 sequence failed to improve the transfection of LAH4-L1 in both RAW264.7 and JAWSII cells. Despite the M9 sequence has been reported to improve nuclear entry in non-viral vectors,[9, 44-46] it was not successful in promoting DNA transfection when it was incorporated into the LAH4-L1. This lack of activity could be due to the fact that NLS failed to be present at the surface of the complexes that render it inaccessible to be recognized by the nuclear transport protein transportin, or the size of complexes were too large (above 300 nm) to be effectively taken up by the cell through endocytosis. In addition, the highest DNA transfection mediated by NQ was obtained at ratio 15:1 (w/w) in all the cell lines tested. Any ratio higher than 15:1 (w/w) was not evaluated in this study. An increased amount of NQ may be required to show any improvement in DNA delivery. Among the three NLSs being investigated, SV40 derived NLS demonstrated the biggest improvement of transfection in all tested cell lines apart from the rapidly dividing A549 cells and there was no significant difference between SV40

derived NLS (PK1) and its reverse sequence (PK2). To gain a better understanding of how SV40 NLS improved DNA transfection, the cellular and nuclear uptake of DNA was evaluated. Peptide/DNA complexes formed at 10:1 ratio (w/w) were selected for the uptake studies due to the good transfection efficiency achieved at this ratio across different cell lines, and the peptides were able to offer protection against nuclease degradation (Fig. S2). Since PK1 and PK2 carry a higher positive charge density than LAH4-L1, and the DNA complexes formed with PK1 and PK2 were more positively charged, as shown in the zeta potential measurement, it is possible that the modified peptides facilitated cellular entry through the non-specific endocytosis more effectively because of the stronger interaction between the cationic complexes and the anionic mammalian cell membranes. Also, PK1, PK2 were able to form sub-200 nm DNA complexes, whereas the complexes formed with LAH4-L1 were just above 200 nm. Nanoparticles below 200 nm are usually more effectively taken up by cells through non-specific endocytosis,[47] which could partly explain why PK1 and PK2 were more efficient in mediating DNA transfection.

To explicitly measure the amount of exogenous DNA inside the nucleus, the nuclei of the transfected cells were isolated. For cells transfected with PK1 and PK2, the amount of exogenous DNA that entered the nucleus was around 1.5-fold and 2-fold higher in RAW264.7 and JAWSII cells respectively, than that transfected with LAH4-L1. The confocal imaging study carried out on Calu-3 cells revealed the co-localization of fluorescently labelled DNA with nucleus in cells transfected with PK2, but not with LAH4-L1, suggesting that nuclear uptake was indeed more efficient with PK2. In addition, cells transfected with LAH4-L1, but not PK2, showed some

co-localization of DNA with lysosomes, suggested that PK2 might be more effective in promoting endosomal/lysosomal escape. Whether the SV40-derived NLS could promote nuclear entry through the interaction with importin remain unknown. Further study is required to examine the intracellular trafficking of DNA delivered by these peptides. In addition, the delivery system could be further optimized by manipulating the number of NLS per molecule of carrier peptides.

CONCLUSIONS

In summary, we demonstrated that the pH responsive LAH4-L1 peptide modified with SV40 derived NLS could significantly enhance DNA transfection in slow dividing cells including airway epithelial cells, macrophages, dendritic cells, as well as thymidine-induced growth-arrested cells. The improved transfection efficiency of these peptides over LAH4-L1 could be due to one, or more likely, the combination of the following reasons: (i) enhanced cellular uptake; (ii) more efficient endosomal/lysosomal escape; (iii) increased transportation across nuclear membrane. The mechanism remains to be elucidated by studying the intracellular trafficking and nuclear transportation of the exogenous DNA. The results of this work suggest that both PK1 and PK2 peptides are promising DNA carriers and further *in vivo* investigation is warranted.

ASSCIATED CONTENT

Supporting Information Table S1 Particle size of peptide/DNA complexes formed at 5:1 and 15:1 ratios (w/w); Table S2 Particle size of peptide/DNA complexes of the combined peptide system; Figure S1 Luciferase DNA transfection study using combined peptide systems; Figure S2 Gel retardation assay of enzymatic degradation.

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Notes The authors declare no competing financial interest.

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ABBREVIATIONS

APC, antigen presenting cells; LDH, lactate dehydrogenase; NLS, nuclear localization signal; NPC, nuclear pore complex; SDS, sodium dodecyl sulphate; SEM, standard error of the mean; SV40, Simian virus 40; TEM, transmission electron microscope.

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Graphic Abstract