Amphiphilic cationic lipopeptides with RGD sequences as gene vectors

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Two kinds of arginine-rich amphiphilic lipopeptides with hydrophobic aliphatic tails ($C_{12}GR_8GDS$, LP1 and $C_{18}GR_8GDS$, LP2) were designed and synthesized as functional gene vectors. With hydrophobic tail modification, these amphiphilic lipopeptides could bind DNA more efficiently and form stable spherical complexes in comparison with the control peptide ($AcGR_8GDS$, P1). Moreover, the size and zeta potential results demonstrated the charge density and stability of the vector/DNA complexes could be improved with the increasing length of the aliphatic tails. *In vitro* transfection experiments showed that LP1 and LP2 could induce much higher gene expression level (luciferase expression) as compared with P1. Due to the incorporation of arginine-glycine-aspartic acid (RGD) sequences which could be specifically recognized by integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ over-expressed on cancer cells, these lipopeptides could be specifically recognized by cancer cells, *i.e.* LP1 and LP2 exhibited relatively higher transfection efficiency in HeLa cell line than that of P2 and P3 without RGD sequence. While the transfection efficiencies of LP2 and P2 were similar in 293T cells. Lipopeptides exhibited very low cell cytotoxicity in both HeLa and 293T cell lines even at high concentration.

Introduction

In recent years, gene therapy have attracted great research interest in curing genetic diseases such as cancer, diabetes and cardiovascular disease.[1,2] The success of gene therapy significantly depends on the availability of suitable delivery vectors, which can be generally divided into viral vectors and non-viral vectors. Because of the relatively low cytotoxicity and ease of preparation, non-viral gene delivery vectors have attracted more attention in comparison with viral vectors.[3,4]

The key issue of designing new non-viral gene vectors is to enhanced the gene delivery capability as well as to improve the biocompatibility.[5–7] In the past few decades, cationic polymers, liposomes, and dendrimers have been developed as non-viral gene carriers with efficient gene delivery capability. However, low biocompatibility and defective bioactivity limit their further applications and developments *in vivo*.[8–11] For instance, polyethylenimine (PEI),[12] especially the 25 kDa branched PEI, can induce efficient gene transfection in many cell lines. However owing to its high toxicity, it is rarely used *in vivo*.[12] Recently, a series of new non-viral gene carriers based on cationic polypeptides and oligopeptides have been reported. Peptides, due to their inherent biodegradability and well biocompatibility, have been widely used in biomedical fields such as tissue engineering, cell culture, and drug delivery. It is known that there are twenty kinds of natural amino acids with different physical and chemical properties because of their different polar/non-polar and acidic/basic/neutral groups, which provide a favorable opportunity to prepare a variety of peptide-based gene carriers. Up to now, poly-L-lysine (PLL) and lysine-, histidine-, and arginine-rich oligopeptides have been developed as gene carriers.[13–17] Among them, the arginine-rich oligopeptides exhibit unique properties to transport through the cell membrane when the number of the sequential arginine residues is between 5 and 11 (the sequences appeared in cell penetrating peptides (CPPs) or protein transduction domains (PTDs)).[18–21] However, due to the relatively lower cationic densities in the molecular structures of arginine-rich oligopeptides compared with the common gene vectors such as PEI, these arginine-based oligopeptides exhibit weak abilities to bind the naked DNA. Therefore, the complexes formed between these arginine-based oligopeptides and naked DNA are not stable enough to transport into nucleus.[22,23] A recent report indicated that the incorporation of hydrophobic cholesterol moieties to the arginine-based oligopeptides can accomplish supramolecular self-assembly to form stable micelles with high cationic density on their surfaces. And it was demonstrated that these micelles can bind with naked DNA to form stable complexes which can transport into nucleus and present comparative level of gene transfection as 25 kDa branched PEI.[24]

Our group recently reported a series of amphiphilic peptides comprised of hydrophilic peptide segments and hydrophobic aliphatic tails, which could self-assemble into stable spherical micelles or fibrous structures with hydrophilic shells and hydrophobic cores in aqueous solutions.[25] In this study, the similar concept was employed by introducing hydrophobic aliphatic chains with different lengths to the structures of arginine-based oligopeptides to form amphiphilic lipopeptides for gene delivery (Scheme 1). In these amphiphilic lipopeptides, octaarginine (R_8), which has been demonstrated to transport through the cell membrane most efficiently, was incorporated into the peptide segments. In addition, the targeted arginine-glycine-aspartic acid (RGD) sequences were also incorporated into the peptide segments of amphiphilic lipopeptides to provide an internalization pathway and enhance targeting cellular uptake through receptor-mediated endocytosis.[26,27] The cytotoxicity assay and *in vitro* transfection study were performed using human cervix carcinoma (HeLa) and human embryonic kidney transformed 293 (293T) cell lines.

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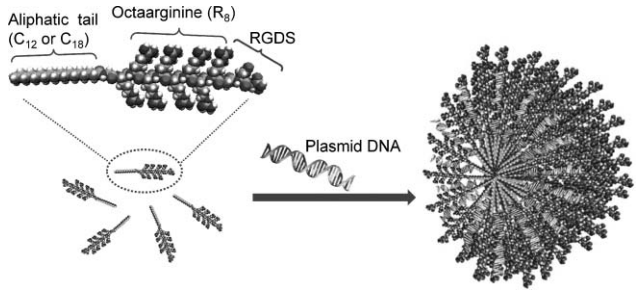


Scheme 1 Complex formed by lipopeptide and DNA.

The data obtained indicated that these amphiphilic lipopeptides could bind with naked DNA to form stable complexes and mediate effective gene transfection. In addition, they could be specifically recognized by abnormal cells. Moreover, the stability of the complexes could be enhanced through increasing the length of the hydrophobic aliphatic chains.

Results and discussion

DNA-binding ability of peptides

The DNA-binding ability of peptides was evaluated by agarose gel electrophoresis assays (Fig. 1). Without any hydrophobic segment modification, P1 had a weak DNA-binding ability, and could hardly retard DNA completely even at a w/w ratio of 20. Lipopeptides with aliphatic tail modification could bind DNA at relatively lower weight ratios. An effective retardation of DNA was achieved at w/w ratio of 15 for lipopeptide LP1, and 10 for LP2. Obviously, the longer the aliphatic tail had conjugated to the peptide the stronger DNA-binding ability it had. The hydrophobic aggregation provided by aliphatic tails of the lipopeptides assisted charge effect between the peptides and DNA. As a hydrophilic peptide, P1 could bind DNA only by the electrostatic interaction,

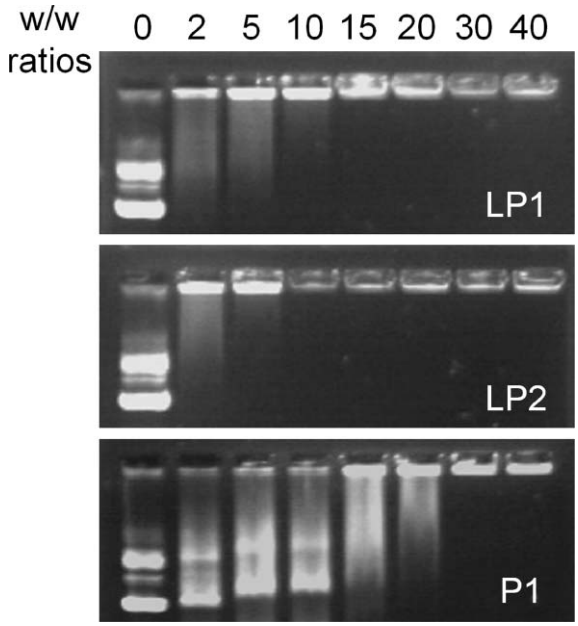


Fig. 1 Agarose gel electrophoresis retardation assay of naked plasmid DNA (Lane 1) and peptide/DNA complexes (lane 2–8) at various w/w ratios as specified.

and the short peptide could not provide adequate force to bind DNA so tight. In comparison with P1, due to the presence of hydrophobic tails in the molecular structures of LP1 and LP2, the aggregation of the hydrophobic tails can appear in aqueous solution. Therefore, besides the electrostatic interactions, the hydrophobic aggregation can also facilitate the binding between LP1 or LP2 and DNA. The stearyl tail possessed a stronger hydrophobic force than the lauryl one, and that was why LP2 had the stronger DNA-binding ability compared with LP1.

Particle size and zeta potential

The average hydrodynamic size and zeta potential of peptide/DNA complexes at different w/w ratios are shown in Fig. 2. The average size of the complexes became small with the increasing w/w ratio when the ratio was lower than 40. It was a significant decline when the w/w ratio raised from 2 to 5. This phenomenon corresponded to the increasing in zeta potential of lipopeptides. The size of P1/DNA complexes was below 250 nm at a high w/w ratio above 30, which showed that P1 was also able to condense plasmids DNA into nanoparticles. The minimum hydrodynamic sizes of the complexes of LP1/DNA and LP2/DNA were about 207 and 197 nm (at w/w ratio of 40), and P1/DNA complex had a minimum size of 243 nm (at w/w ratio of 40). Furthermore, both average sizes of LP1 and LP2 were much smaller than P1. It demonstrated that octaarginine residue provided the capability to condensed DNA and the hydrophobic tail enhanced this effect.

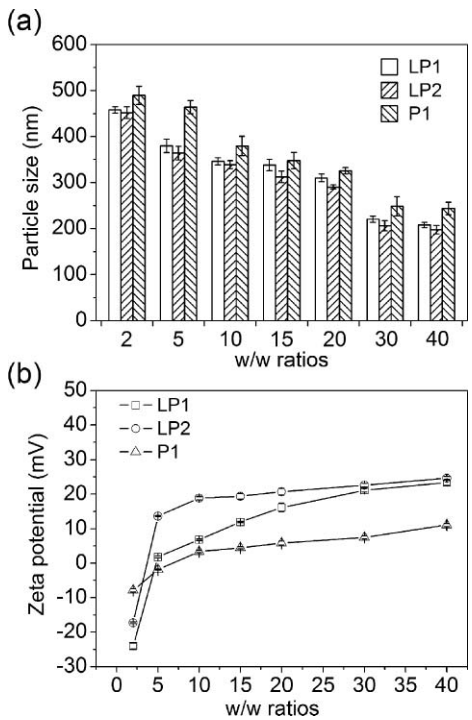


Fig. 2 (**a**) Size (average diameter) and (**b**) zeta potential of peptide/DNA complexes at various w/w ratios as specified. Error bars represent standard deviation of 6 replicates for the test.

The zeta potential of binary complexes showed an increasing trend with the increase in w/w ratios. After the modification of fatty tails, the surface charge of the complexes became positive at w/w ratio of 5. Peptides became amphiphilic due to the

conjugation of hydrophobic tails, which made complexes become more condense. The hydrophobic tails, the lauryl one and the stearyl one provided the corresponding lipopeptides a stronger DNA-binding ability, so LP1 and LP2 could shield negative charge of DNA at lower w/w ratios. Due to the increased density of cationic charge, LP1 and LP2 exhibited enhanced DNA condensation. The maximum zeta potentials of the complexes formed by LP1 and LP2 with DNA were 23.4 and 24.7 mV respectively, which were much higher than that of P1/DNA. As higher positive zeta potential might to some extent promote cellular uptake, the cationic complexes formed by lipopeptide might deliver DNA to cell more efficiently.

Morphology analysis

The morphology of peptide/DNA complexes observed by TEM was shown in Fig. 3. The complex sizes were much smaller than the sizes measured by dynamic light scattering (DLS), which was due to the shrinking of nanoparticles during the preparation of TEM samples. It was evident that some homogeneous and stable spherical complexes formed by mixing LP2 with DNA at the assigned w/w ratio. As the result of LP1 and P1 could not fully bind DNA at this w/w ratio, spherical complexes formed by these two peptides were at different sizes. This situation was obvious in P1/DNA complex system. The zeta potential of P1/DNA complex at the w/w ratio of 15 was 4.45, and the charge was too weak to prevent from complexes aggregating together, this phenomenon was clearly illustrated by TEM image.

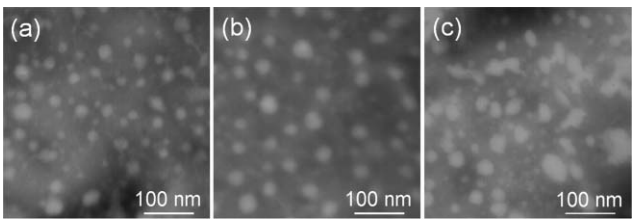


Fig. 3 TEM images of peptide/DNA complexes at w/w ratios of 15. (**a**) LP1/DNA, (**b**) LP2/DNA and (**c**) P1/DNA.

Cytotoxicity of peptides

The cytotoxicity of peptides was tested against HeLa and 293T cell lines by MTT assay, and the 25kDa PEI was used as control. From Fig. 4, it was found the cytotoxicity of peptides increased with the increasing w/w ratio in both cell lines. However, it was still very low in comparison with PEI. In HeLa cells, two lipopeptides and P1 did not showed apparent cytotoxicity. The relative cell viabilities for LP1 and LP2 at 200 mg L^{-1} were about 80 and 83%. In 293T cells, LP1 and P1 still kept their low cytotoxicity, and the cell viabilities at 200 mg L^{-1} were about 89% and 90%, respectively. However, LP2 exhibited a slight cytotoxicity at a high concentration, and the cell viability at 200 mg L^{-1} was about 73%. This situation may correlate with charge concentration. With the modification of hydrophobic tail, especially the stearyl one, lipopeptides would form some analogs of micelle as the concentration increased, and thus the local density of positive charge increased as well, which might to some extent cause membrane disruption, and eventually led to cytotoxicity to cells.

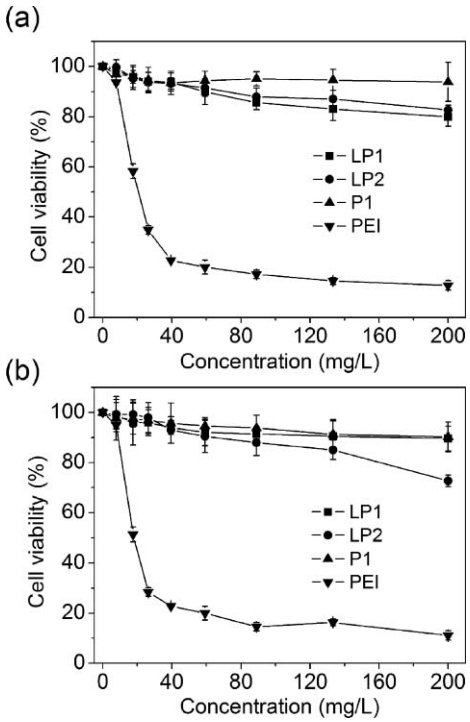


Fig. 4 Cytotoxicity of LP1, LP2 and P1 in (**a**) HeLa cells and (**b**) 293T cells, 25 kDa PEI as control. Error bars represent standard deviation of 4 replicates for the test.

Gene transfection

The luciferase expression efficiency mediated by peptides was evaluated in HeLa and 293T cell lines at various w/w ratios, and PEI as control performed at its optimal w/w ratio of 1.33 as shown in Fig. 5. The luciferase expression efficiency was influenced by cell type and w/w ratios. In HeLa cells, the peak values of luciferase expression levels induced by LP1, LP2 and P1 were 8.98×10^7 RLU/mg protein (w/w 15), 9.59×10^7 RLU/mg protein (w/w 10) and 4.90×10^5 RLU/mg protein (w/w 5) respectively, and by PEI was 1.14×10^8 RLU/mg protein. And in 293T cells, the peak values of luciferase expression levels induced by LP1, LP2 and P1 were 6.20×10^7 RLU/mg protein (w/w 10), 2.59×10^8 RLU/mg protein (w/w 10) and 5.66×10^5 RLU/mg protein (w/w 2) respectively, and by PEI was 6.47×10^8 RLU/mg protein. It was obvious that the fatty tail modification promoted gene delivering to cells effectively. The luciferase expression efficiency induced by lipopeptides increased significantly compared to peptide P1, and this phenomenon was observed in both HeLa and 293T cell lines. With the w/w ratios increased from 2 to 10, the luciferase expression level increased because of the promoted cellular uptake at a higher w/w ratio since zeta-potential increased and size decreased with increasing w/w ratio. However, with the further increased w/w ratio, the gene transfection efficiency of LP1 and LP2 decreased. This phenomenon might be caused by the strong binding between the vector and DNA, and thus the DNA was not able to escape from the complexes to express in nucleus. On the other hand, after DNA was fully bound by the peptide, the excess peptide molecules gathered around or self-assembled into nanoparticles induced by hydrophobic force. These nanoparticles had the same capability of adhering to cell and penetrating through

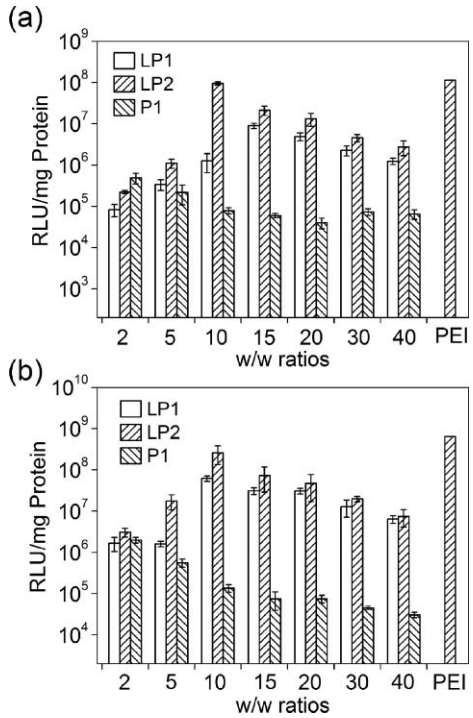


Fig. 5 *In vitro* luciferase expression level mediated by LP1, LP2 and P1 in (**a**) HeLa cells and (**b**) 293T cells. PEI/DNA performed at the optimal w/w ratio of 1.33 as the control. Error bars represent standard deviation of 3 replicates for the test.

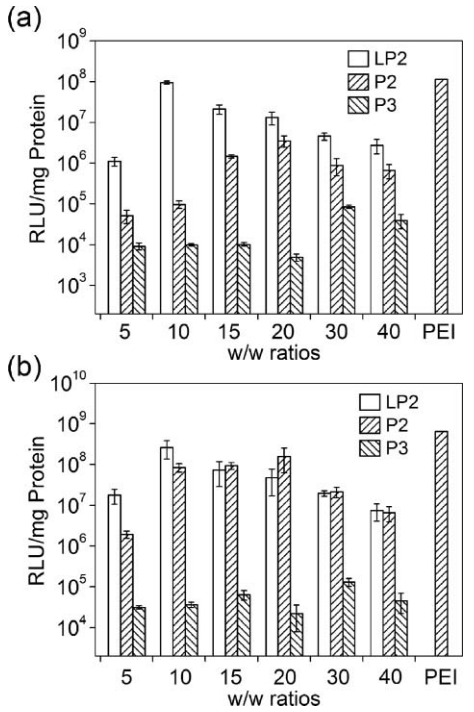


Fig. 6 *In vitro* luciferase expression level mediated by LP2, P2 and P3 in (**a**) HeLa cells and (**b**) 293T cells. PEI/DNA performed at the optimal w/w ratio of 1.33 as the control. Error bars represent standard deviation of 3 replicates for the test.

the cell membrane, so they might compete with complexes to pass though cell membrane and to some extent decreased the gene expression efficiency. As the DNA binding ability of P1 was weak, there was not much difference in the luciferase expression at w/w ratios from 5 to 40, which was in accordance with the results showed by zeta potential measurement.

Targeting affection of RGD sequence in gene transfection

Target ligands, such as folic acid,[28,29] transferrin[30–32] and the well known tripeptide sequence arginine-glycine-aspartic acid (RGD),[33–37] have been widely employed to conjugate with vectors for specific recognition and increased uptake into the target cells. The RGD peptide residue which can be recognized by integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, plays a significant role in the adhesion and migration of tumor cells.[38,39] The function of RGD sequence in gene transfection was evaluated by comparing the transfection efficiencies of LP2, stearyl-octaarginine ($C_{18}R_8$, P2) and R_8 (P3) in HeLa and 293T cell lines (Fig. 6). In HeLa cells, the luciferase expression level induced by P2 was much higher than P3, indicating the promotion of fatty tail in gene delivery. While LP2 performed much better than P2. However, in 293T cells, the luciferase expression levels induced by P2 and P3 were much higher than that in HeLa cells. Moreover, P2 could even perform better than LP2 at its optimal w/w ratio. Overall, the difference between LP2 and P2 in gene delivery tested against 293T cells was unobvious compared to that tested in HeLa cells. This phenomenon indicated the function of RGD sequence in specific recognition. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, those serve as receptors for extracellular matrix protein with exposed RGD sequence, always express at a very low level on mature endothelial cells and epithelial cells, nevertheless they express overdose on activated endothelial cells and some tumor cells, such as glioblastomas, neuroblastomas and breast cancer.[40,41] So in HeLa cell line, a tumor cell, lipopeptides with RGD could induce much higher luciferase expression level than P2. As integrins expressed at very low level on 293T cells, sequence RGD could not play the role. Consequently, these lipopeptides, which had good bioactivity in specific recognition, could efficiently mediate gene transfection.

Conclusions

Two kinds of lipopeptides LP1 and LP2 were synthesized. With the modification of hydrophobic aliphatic tail, the lipopeptides could bind DNA more efficiently at lower w/w ratios, and could induce much higher gene transfection efficiency in both HeLa and 293T cell lines with comparison to the control peptide P1. In addition, the introduction of RGD sequence into lipopeptide yielded an obvious increase in gene expression level in HeLa cells, indicating these lipopeptides had good specific recognition to abnormal cells. The results of *in vitro* transfection demonstrated that the lipopeptides had similar gene expression efficiency compared with 25 kDa PEI, while they showed much lower cytotoxicity than PEI. These lipopeptides could be used as promising vectors in gene therapy.

Experimental

Materials

N-Fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH and

Fmoc-Ser(tBu)-OH), 2-chlorotrityl chloride resin (100–200 mesh, loading: 0.4 mmol g^{-1}, 1%DVB), *o*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU), and piperidine were purchased from GL Biochem Ltd. (Shanghai, China) and used as received. Trifluoroacetic acid (TFA), phenol, 1,2-ethanedithiol (EDT), lauric acid, and stearic acid were provided by Shanghai Reagent Chemical Co., (China) and used directly. Thioanisole and polyethylenimine (branched PEI, Mw 25 kDa) were purchased from Acros and Sigma-Aldrich, respectively and used without further purification. Diisopropylethylamine (DiEA), *N*,*N*′-dimethylformamide (DMF) and dichloromethane (DCM) were provided by Shanghai Reagent Chemical Co., (China) and distilled prior to use.

QIAfilter™ plasmid purification Giga Kit (5) was purchased from Qiagen (Hilden, Germany). GelRed™ was provided by Biotium (CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin, trypsin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), and Dulbecco's phosphate buffered saline (PBS) were purchased from Invitrogen Corp. The Micro BCA protein assay kit was purchased from Pierce. All other reagents and solvents were used before further purification.

Synthesis of peptides

All the peptides, $C_{12}GR_8GDS$ (LP1), $C_{18}GR_8GDS$ (LP1), $AcGR_8GDS$ (P1), $C_{18}R_8$ (P2), and R_8 (P3) (Scheme 2) were synthesized manually employing a standard Fmoc chemistry.[42] Peptide chains were grown on 2-chlorotrityl chloride resin. The coupling of the first residue used 4 equiv. (relative to the substitution degree of resin) Fmoc-protected amino acid and 6 equiv. of DiEA in a DMF solution for 2 h. Other amino acid couplings were carried out with 4 equiv. of Fmoc-protecting amino acid, 4 equiv. of HBTU, and 6 equiv. of DiEA for 4 h. During the synthesis, Fmoc protected groups were deprotected with 20% piperidine/DMF (v/v) for twice. At the end of the synthesis, the fatty tails were conjugated to the peptide segments after activating the aliphatic acids by HBTU and DiEA in a DMF–DCM mixed solution. After the completion of the synthesis, the resin was finally washed with DMF (three times) and DCM (three times) and dried under vacuum for 24 h. Cleavage of the expected peptides and the removal of side chain protected groups from the dried resin were performed by suspending the resin in a cleavage cocktail containing TFA (83%), phenol (6.3%), thioanisole (4.3%), H_2O (4.3%), and EDT (2.1%) for 2 h. The filtration was concentrated to a viscous solution by rotary evaporation. After the precipitation in cold ether, the crude product was collected and vacuum dried, then dissolved in distilled water and freeze-dried. The molecular weights of LP1, LP2, P1, P2 and P3 found in ESI-MS (LCQ Advantage, Finigan, USA) were 884.1 $[M+2H]^{2+}$, 926.2 $[M+2H]^{2+}$, 813.7 $[M+2H]^{2+}$, 774.9 $[M+2H]^{2+}$and 634.7 $[M+2H]^{2+}$ respectively (Table 1).

Cell culture and amplification of plasmids DNA

HeLa and 293T cells were incubated in DMEM medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10,000 U/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

Table 1 Molecular weight of peptides measured by ESI-MS

	Peptide Sequence	$[M+2H]^{2+}$.[a]	Molecular Weight[b]
LP1	C_{12}GRRRRRRRRGDS	884.1	1766.1
LP2	C_{18}GRRRRRRRRGDS	926.2	1850.2
P1	AcGRRRRRRRRGDS	813.7	1625.8
P2	C_{18}RRRRRRRR	774.9	1548.0
P3	RRRRRRRR	634.7	1267.5

[a] $[M+2H]^{2+}$ were found in ESI-MS spectrum. [b] Theoretical molecular weight.

pGL-3 plasmid used in this study as the luciferase reporter gene was transformed in *Escherichia coli* JM109. The plasmid was amplified in terrific broth media at 37 °C overnight, and purified by an EndoFree QiAfilter Plasmid Giga Kit (5). Then the purified plasmid was dissolved in TE buffer and stored at –20 °C. The integrity of plasmid was confirmed by agarose gel electrophoresis, the purity and concentration of plasmid was determined by ultraviolet (UV) absorbance at 260 and 280 nm.

Preparation of peptide/DNA and PEI/DNA complexes

The peptides and PEI were dissolved in 150 mM NaCl buffer and ultra pure water (HPLC grade) respectively. The complexes were prepared by mixing peptides or PEI solution with 1.0 μl pGL-3 DNA (100 ng μl^{-1} in 40 mM Tris-HCl buffer solution) directly at different weight ratios (w/w) beforehand, and then diluted to a total volume of 100 μl with 150 mM NaCl and vortexed for 5 s. The complexes were incubated at 37 °C for 30 min upon mixing before being used for further studies.

Agarose gel electrophoresis assay

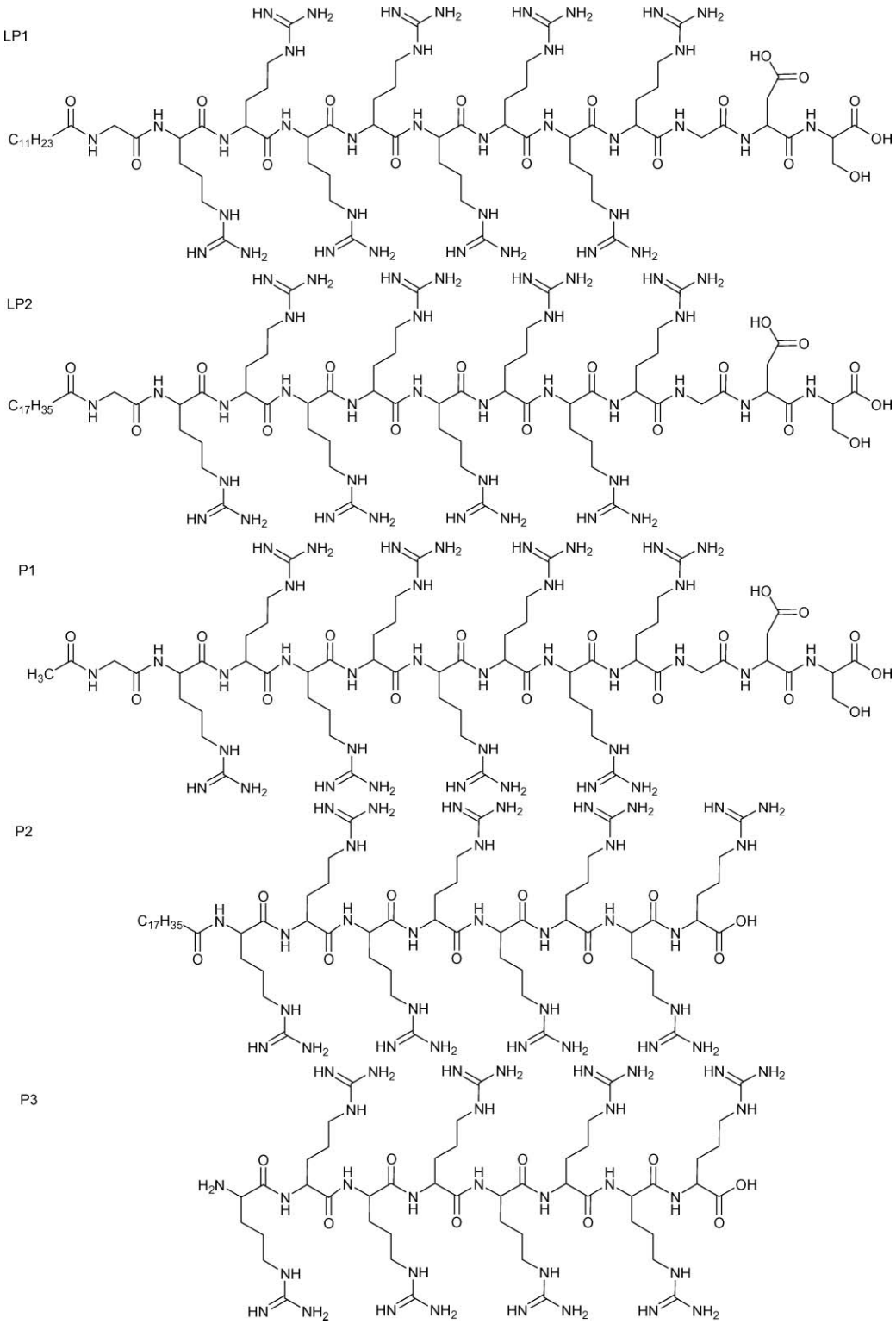
The peptide/DNA binary complexes were prepared at varying weight ratios ranging from 2 to 40 by adding appropriate volumes of peptide (in 150 mM NaCl solution) to 100 ng of pGL-3 DNA (100 ng μl^{-1} in 40 mM Tris-HCl buffer solution). The complexes were diluted to a total volume of 7 μl with 150 mM NaCl solution, and then the complexes were incubated at 37 °C for 30 min. After that the complexes were electrophoresed in the 0.7% (w/v) agarose gel containing GelRed and with Tris-acetate (TAE) running buffer at 70 V for 60 min. DNA was visualized with a UV lamp using a Vilber Lourmat imaging system (France).

Particle size and zeta potential measurement

The particle size and zeta potential were measured on a Nano-ZS ZEN3600 (MALVERN Instruments) at 25 °C. The peptide/DNA binary complexes were prepared at w/w ratios ranging from 2 to 40. Then the prepared complexes were diluted by ultra pure water to 1 ml volume for the size and zeta potential measurements. The measurements were repeated 6 runs for each sample, and the data were reported as the average of readings.

Transmission electron microscopy (TEM)

Morphology observation of peptide/DNA complexes were carried out on a JEM-100CX II instrument operating at an acceleration voltage of 80 kV. The sample was prepared by dipping a copper grid with formavar film into the peptide/DNA complexes at the w/w ratio of 15. A few minutes after the deposition, excess solution



Scheme 2 Chemical structure of peptides.

was blotted away and stained with phosphotungstic acid aqueous solution and then dried in air.

In vitro cytotoxicity assay

The cytotoxicity assay was performed with HeLa and 293T cells by MTT assay. Briefly, the HeLa and 293T cells were seeded respectively in 96-well plates at a density of 6000 cells/well, and then cells were incubated in 100 μl DMEM containing 10% FBS for 1 day prior to adding the peptides. After the peptides were added for 2 days, the medium was replaced with 200 μl of fresh medium. Then 20 μl MTT (5 mg ml^{-1} in PBS buffer) solution was added to each well and further incubated for 4 h. After

that, the medium was removed and 150 μl DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated as: cell viability (%) = $(OD_{570\,(sample)}/OD_{570\,(control)}) \times 100$, where $OD_{570\,(control)}$ was obtained in the absence of peptides and $OD_{570\,(sample)}$ was obtained in the presence of peptides. Each value was averaged from four independent experiments.

In vitro gene expression

For gene transfection experiment (luciferase expression), the pGL-3 plasmid DNA was used to evaluate the transfection efficiency of peptides, and the 25 kDa PEI was used as the positive control. The HeLa cells and 293T cells were seeded in 24-well plates at a density of 6×10^4 cells/well and cultured with 1 ml DMEM containing 10% FBS for 1 day respectively. The transfection efficiency of peptide/DNA complexes were evaluated at various w/w ratios ranging from 5 to 40, and then incubated at 37 °C for 30 min. Then the complexes were added into the plate with serum-free DMEM for 4 h at 37 °C. After that, the serum-free DMEM was replaced with fresh DMEM containing 10% FBS and the cells were further incubated for 2 days. For luciferase assay, the medium was removed and cells were washed by PBS, then the cells were lysed using 200 μl reporter lysis buffer (Pierce). The relative light units (RLUs) were measured with chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein was measured according to a BCA protein assay kit (Pierce) and luciferase activity was expressed as RLU/mg protein.

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