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Supplementary data

Self-assembly of bi-functional peptides on large-pore mesoporous silica nanoparticles for miRNA binding and delivery

Jingxiong Lu,[a] Hsin-Hui Shen,*[b] Zhangxiong Wu,[c] Bo Wang,[d] Dongyuan Zhao[a,e] and Lizhong He*[a]

[a] *Department of Chemical Engineering, Monash University, Wellington road, Melbourne 3800, Australia E-mail: lizhong.he@monash.edu*

[b] *Department of Microbiology, Monash University, Wellington road, Melbourne 3800, Australia E-mail: hsin-hui.shen@monash.edu*

[c] *College of Chemistry Chemical Engineering and Materials Science, Soochow University, Suzhou, Jiangsu 215123, P. R. China*

[d] *Department of Anatomy and Developmental Biology, Monash University, Wellington road, Melbourne 3800, Australia*

[e] *Department of Chemistry, Shanghai Key Laboratory of Molecular Catalysis and Innovative Materials, and Laboratory of Advanced Materials, Fudan University, Shanghai 200433, P. R. China*

Materials and methods

1. Materials

All the peptides were custom synthesized by Mocell Biotech Co., Ltd (Shanghai, China), and the physical properties of peptides are summarized in Table S1. Tetraethylorthosilicate (TEOS), 3,3',5,5'-Tetramethylbenzidine (TMB), Pluronic® F-127 and MSNs with a size of around 500 nm and pore size of 4 nm was purchased from Sigma Aldrich (St. Louis, MO, USA). Fluorocarbon surfactant FC-4 was from Yick-Vik (Hong Kong, China). CpG DNA 18263 (TCCATGACGTTCCTGACGTT), Cy3™ Dye-Labeled Pre-miR Negative Control #1, miR-29b-3p and Turbo DNA-free kit were obtained from Life Technologies (Carlsbad, CA, USA). Faststart universal SYBR green master and transcription high fidelity cDNA synthesis kit were

purchased from Roche (Basel, Switzerland). Normal rat kidney (NRK) cell for transfection experiments was a generous gift from Dr. Phillip Kantharidis, Baker IDI Heart and Diabetes Institute.

2. Synthesis of silica nanoparticles

The large-pore mesoporous silica nanoparticles (LP-MSNs) were synthesized following the method reported by Gao et al.[1]. Slight modifications are described below; otherwise the experimental conditions were the same as reported in the literature. In the first hydrothermal treatment step, a temperature of 150 °C was chosen in order to achieve large pores. The products were filtered, and then sequentially washed with water and ethanol before being dried in air. This was followed by the second hydrothermal treatment using the same conditions suggested by Gao et al. Template was removed by adding 0.5 g of the as-prepared product into 100 mL of absolute ethanol, followed by addition of 2 mL of (2 M) HCl. Then, with running water as coolant, the solution was refluxed at 80 °C for 48 h in a water bath. The final products were washed with water and ethanol, and then dried in air at 60 °C in the oven.

The as prepared LP-MSNs were characterized by FEI Nova NanoSEM 450 and FEI Tecnai G2 T20 TEM at Monash Centre for Electron Microscopy (MCEM). Non-porous solid silica nanoparticles with a diameter of 200 nm were synthesized by the Stöber method.[2]

3. Adsorption of CpG DNA into silica nanoparticle-peptide complexes

Silica nanoparticle-peptide complexes were formed by self-assembly of 1 mL 2 mg/mL peptide solution onto 1 mg silica nanoparticles for 15 min, as illustrated in figure S1. CpG DNA 1826 (TCCATGACGTTCCTGACGTT) [3] was employed as a mimic of miRNA for in vitro nucleic acid adsorption study. Both DNA and peptide concentrations were examined for their effect on DNA adsorption. In the first set of experiments, three types of silica nanoparticles were compared for adsorption of CpG DNA. The effect of concentration of peptide RGRRRRLSCRLLK$_8$ on DNA adsorption was examined at a fixed concentration of CpG DNA. Briefly, 0.1 mg of each type of silica nanoparticle was mixed with 0.1 ml of the peptide at concentration of 10, 20, 40, 80, 160, 320 and 640 μg/ml for 15 minutes to form particle-peptide complex by self-assembly of peptides on silica nanoparticles. After centrifugation at 9000 g for 3 min, supernatant was removed and 50 μl 0.1 mg/ml CpG DNA was added to the remained particles-peptide complexes. After 15 min

incubation, the particles were settled down by centrifugation (9000 g for 3 min) and the supernatant was taken for analysis. In the second set of experiments, isotherm of CpG DNA adsorption into LP-MSN-peptide complex was tested at a fixed peptide concentration for three different peptides. 3 mg of LP-MSNs were mixed with 3 mL of 2 mg/mL peptide for 15 min to form LP-MSN-peptide complexes. After removal of supernatant, 0.3 mg LP-MSN-peptide complexes were mixed with 50 μL DNA aqueous solutions at the concentration of 0, 10, 20, 40, 60, 80, 100, 300, and 500 μg/mL for 15 min. The concentrations of DNA solutions after adsorption were measured by absorbance A_{260} using Nanodrop 1000 (Thermo scientific, Australia).

4. Protection of nucleic acid loaded into LP-MSN from DNase I digestion

10 μl of 100 μg/ml CpG DNA, loaded into 50 μg of LP-MSN- RGRRRRLSCRLLK8 or not, mixed with 0.02, 0.2 and 2 U DNase I. After incubation at 37 °C for 1 h, the digested DNA was examined by 20 % acrylamide gel run under 200 V for 45 min. The TBE-PAGE electrophoresis results are presented in Figure S2.

5. Cell experiments

5.1 Delivery efficiency of LP-MSN-peptide complexes

For the following experiments, the LP-MSN-peptide complexes were typically formed by mixing 1 mg silica particles with 1 mL 2 mg/mL peptide solution. To determine the uptake of gene adsorbed LP-MSN-peptide complexes into NRK cells, a fluorescent labeled miRNA, Cy3™ Dye-Labeled Pre-miR Negative Control #1 was used as the transfection indicator. NRK cells were seeded on a coverslip (18 × 18 mm² with a thickness D =0.17mm) at density of 10,000 cells/well in a 6-well plate. After 24 h, 50 μg LP-MSN-peptide complexes were mix with 10 μL 5μM Cy3 labeled miRNA for 15 min at room temperature (RT). Then the fluorescent miRNA adsorbed on LP-MSN-peptide complexes were dispersed in 2 mL complete Dulbecco's Modified Eagle's Medium (DMEM) before adding into the well. The delivery efficiency of Lipofectamine 2000 (Life Technology, USA) was also tested according to the manufacturer's manual. For negative control, 10 μL 5μM Cy3 labeled miRNA without LP-MSN-peptide complexes was dispersed in 2 mL DMEM and added into the well. After 2 hours' incubation, cells were fixed by 5% paraformaldehyde for 5 min. After washing the fixed cells with phosphate buffered saline (PBS) twice, the filament actin was stained by FITC-Phalloidin (Sigma, Sydney, Australia) and the nuclear staining was performed by Hoechst (Thermo Scientific, Melbourne,

Australia). Fluorescence image of the samples were taken using a fluorescent confocal microscopy SP5 (Leica, Germany) at Monash Micro Imaging (MMI).

5.2 Evaluation of biological function of miRNA delivered by LP-MSN-RGRRRRLSCRLLK8

A functional miRNA (miR-29b-3p) was used to detect the function of delivered miRNA by LP-MSN-RGRRRRLSCRLLK$_8$ complexes. NRK cells were pre-seeded in a 6-well plate (10,000 cells/ well) for 24 h. 50 μg LP-MSN-peptide complexes were mix with 10 μL 5μM miR-29b-3p for 15 min. Then the mixed solutions were dispersed in 2 mL complete DMEM before replacing the medium in each well. The function of miR-29b-3p delivered by commercial Lipofectamine 2000 was also evaluated following the manufacture's manual. Control group was set using normal cells without miRNA delivery. 24 hours later, the culture medium was refreshed. After another 24 hours, total RNA was harvested and purified by Turbo DNA-free kit. The reverse transcription was performed to obtain cDNAs using transcription high fidelity cDNA synthesis kit. Quantitative PCR were run by mixing cDNA and primers with faststart universal SYBR Green master. The expression levels of miR-29b-3p down-regulated genes collagen 1, collagen 3, collagen 4, and collagen 4 alpha 3 were detected. GAPDH gene was employed to standardize the results using normal cells as the control group.

Primers used for qPCR:

Collagen 1 forward: 5'-TGCCGATGTCGCTATCCA-3';

Collagen 1 reverse: 5'-TCTTGCAGTGATAGGTGATGTTCTG-3';

Collagen 3 forward: 5'-GGAAAAGATGGATCAAGTGGACAT-3';

Collagen 3 reverse: 5'-GAGCCCTCAGATCCTCTTTCA-3';

Collagen 4 forward: 5'-CACTATGAAAACCGTAAAGTGCCTTA-3';

Collagen 4 reverse: 5'-GCAAACAGAGGCCAACGAA-3';

Collagen 4 alpha 3 forward : 5'-CAAACCACAGCCAATCCTTCA-3' ;

Collagen 4 alpha 3 reverse : 5'-AAGAAGGGAAAACCCACTGTAGAGT-3' ;

GAPDH forward : 5'-GGCACAGTCAAGGCTGAGAATG-3' ;

GAPDH reverse : 5'-ATGGTGGTGAAGACGCCAGTA-3'.

5.3 Cytoxicity

The cytotoxicity of LP-MSN-RGRRRRLSCRLLK$_8$ and commercial transfectant Lipofectamine 2000 was determined by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. NRK cells were pre-seeded in a 96-well plate (3,000 cells/ well) and triplicates were carried out for each sample. After 24 h, the medium in the wells was aspirated out and 100 μL of each LP-MSN-peptide complexes at a series of concentration or 0.5 μL of Lipofectamine 2000 in 100 μL complete DMEM were added into each well. Normally cultured cells were set as control. After another 24 h, the culture medium was removed, followed by gently rinsing the substrate with PBS three times. 100 μL 0.5 mg/mL MTT in DMEM was added into each well. At 100% humidity atmosphere with 5% CO_2, the cells were incubated at 37.5 °C for 4 h. After removing the supernatant, 100 μL dimethyl sulfoxide (DMSO) (Sigma, Australia) was introduced to each well. After the dissolution of the purple formazan by incubating at 37.5 °C for 10 min, the absorbance of supernatant solution was determined at 540 nm.

6. Size of peptides and miRNA

The mean contour length of double stranded RNA was reported to be 0.29 ±0.03 nm per base.[25] The length of miRNA of 22 bases was thus estimated to be 6.4 ± 0.7 nm. The length scale of silica binding peptides was reported to be 2-3 nm.[18] Taking into account of poly-lysine, the length scale of bi-functional peptides was estimated to be 3-5 nm.

Table S1. Physical properties of peptides.

Peptides	Molecular weight [a)]	Molecular volume [b)]	Peptide length [c)]	pI [d)]
RGRRRRLSCRLL	1541.8 (1540.9)	1.86 nm^3	3-5 nm	12.30
RGRRRRLSCRLLK8	2567.2 (2566.30)	3.27 nm^3	3-5 nm	12.31
KSLSRHDHIHHHK8	2529.0 (2529.06)	3.25 nm^3	3-5 nm	10.87

[a)] Theoretical and experimental values of molecular weight. The values in bracket were determined by mass spectroscopy.

[b)] Molecular volume was estimated from amino acid sequence.[6]

[c)] The silica binding peptides without K_8 have a length scale of 2-3 nm by molecular dynamics simulation,[5] and the full length including K_8 was thus estimated to be 3-5 nm.

[d)] Isoelectronic point, pI, was calculated using ProtParam.[7]

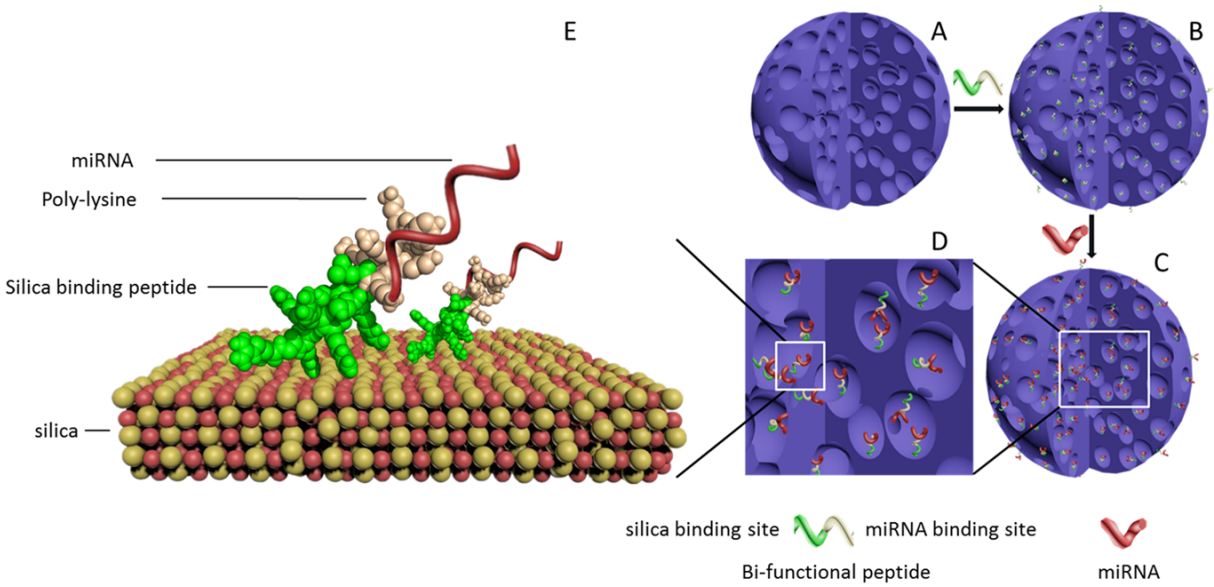

Figure S1. Schematic illustration of the preparation of LP-MSN-peptide-miRNA complex. A: LP-MSN; B: LP-MSN-peptide complexes that are formed by self-assembly of biofunctional peptides; C: LP-MSN-peptide-miRNA complex; D: Adsorption of peptide-miRNA inside of the mesopores; E: bi-functional peptides self-assemble on silica through silica binding moiety and miRNA is bound by poly-lysine moiety.

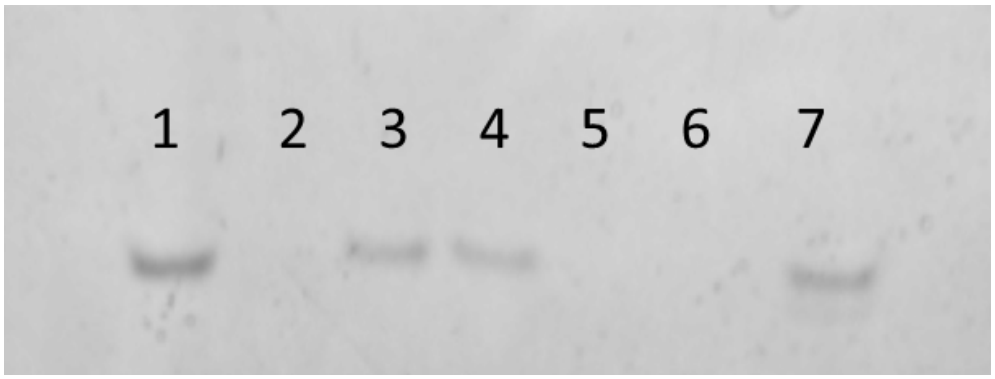

Figure S2. TBE-PAGE electrophoresis for determination of CpG DNA integrality after DNase I treatment. Lane 1: CpG DNA without digestion; lane 2: CpG DNA loaded into LP-MSN- RGRRRRLSCRLLK$_8$ then digested by 2 U DNase I; lane 3: CpG DNA loaded into LP-MSN- RGRRRRLSCRLLK$_8$ then digested by 0.2 U DNase I; lane 4: CpG DNA loaded into LP-MSN- RGRRRRLSCRLLK$_8$ then digested by 0.02 U DNase I; lane 5: CpG DNA digested by 2 U DNase I; lane 6: CpG DNA digested by 0.2 U DNase I; lane 7: CpG DNA digested by 0.02 U DNase I.

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