Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

A helix foldamer oligopeptide improves intracellular stability and prolongs protein expression of delivered mRNA

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Materials and Methods

Materials

OligoArg and OligoArg-Aib were synthesized as previously described.[1] Briefly, the peptides were synthesized on a solid support by Fmoc solid-phase methods using standard commercially available Rink amide resin and Fmoc amino acids. The synthesized peptides were purified by reverse-phase high-performance liquid chromatography and characterized by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. *Firefly luciferase* (*fLuc*) mRNA without chemical modification was purchased from Trilink BioTechnologies (San Diego, CA, USA). A template plasmid for preparing destabilized luciferase was constructed from pGL4.12 plasmid (Promega, Madison, WI, USA) containing the *Luc2CP* sequence and the pSP73 plasmid (Promega) containing the 120 nt polyA/T sequences for polyA tailing. The *Luc2CP* sequence was cloned into the pSP73 plasmid at the site between the T7 promoter and polyA/T sequence. *In vitro* transcription was performed using the mMessage mMachine T7 Transcription Kit (ThermoFisher, Waltham, MA, USA).

Dynamic light scattering (DLS) measurement

Oligopeptides and mRNAs were dissolved in HEPES buffer (pH 7.3), and then mixed with each other at [guanidino groups in oligopeptides (N)] / [phosphate groups in mRNA (P)] ratios of 2 or 4. The size and size distribution of the oligopeptide/mRNA polyplexes in HEPES buffer (pH 7.3) were evaluated by DLS using a Nano ZS (ZEN3600, Malvern Instruments, Ltd., UK) with an incident light of 633 nm. Measurements were carried out with a detection angle of 173° and temperature of 37 °C, and the data were subsequently analyzed by the cumulant method.

Measurement of cell viability and luciferase expression

A human hepatoma-derived cell line (HuH-7, RIKEN Cell Bank, Tsukuba, Japan) was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., Madison, WI, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich Co.) and 1% penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan). A total of 5,000 cells were seeded per well in 96-well plates. Twenty-four hours after the incubation, the medium was replaced with fresh medium, and an oligopeptide/mRNA polyplex containing 0.25 μg of mRNA was added to each well. After evaluation of cell viability using Cell Counting Kit 8 (Dojindo Laboratories, Kumamoto, Japan), cells were lysed using a passive lysis buffer (Promega) and subjected to a luciferase assay using the Luciferase Assay System (Promega) and Lumat3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Nuclease stability in serum

Oligopeptide/mRNA polyplexes were incubated in 10 % FBS with a final mRNA concentration of 8.3 μg/mL. After 15 min of incubation, RLT buffer provided in the RNeasy Mini Kit (Qiagen, Hilden, Germany) and supplemented with β-mercaptoethanol was added to stop nuclease activity. This was followed by mRNA purification, which was carried out using the RNeasy Mini Kit, and reverse transcription, performed using the ReverTra Ace qPCR RT Master Mix kit (Toyobo Life Science, Osaka, Japan). Complementary DNA was subjected to quantitative PCR (qPCR) using a primer pair (forward: CAAGAAGGGCCTGCAGAAGA, reverse: GCTGGTCACGAAGGTGTACA), SYBR Green Master Mix (Roche, Basel, Switzerland), and the Mic qPCR Cycler (Bio Molecular Systems, Queensland, Australia).

Measurement of mRNA amount inside cells

A total of 30,000 cells/well were seeded onto 24-well plates and incubated for 24 h. After the medium was replaced with fresh medium, polyplexes containing 1.5 μg of mRNA were added to each well. At the indicated time points, cells were washed with phosphate-buffered saline (PBS), followed by lysis using the RLT buffer provided in the RNeasy Mini Kit (Qiagen, Hilden, Germany), supplemented with β-mercaptoethanol. Then, mRNA purification, reverse transcription, and qPCR were performed as

described above. For evaluation of intracellular mRNA stability, 30 min after mRNA addition, cells were washed twice with PBS to stop cellular uptake of mRNA and then added with fresh medium for an additional 4 or 24 h of incubation. After incubation, the amount of mRNA in the cells was quantified as described above. The obtained values were divided by those obtained from cells immediately after 30 min of incubation with oligopeptide/mRNA complexes to evaluate intracellular mRNA stability.

Measurement of oligopeptide amount inside cells

A total of 30,000 cells/well were seeded onto 24-well plates and incubated for 24 h. After medium exchange, polyplexes containing 1.5 μg of mRNA were added to each well. Thirty minutes after mRNA addition, cells were washed twice with PBS and fresh medium was added to stop additional polyplex cellular uptake. Four or 24 h after stopping polyplex uptake, cells were collected for fluorescence observation using flow cytometry RF-500 (Sysmex, Hyogo, Japan). The obtained fluorescence intensity values were normalized to those obtained from cells immediately after 30 min of incubation with oligopeptide/mRNA complexes.

Fluorescence microscopic observation

mRNA was labeled with Cy5 using the Label IT Tracker™ Cy5 Kit (Mirus, Madison, WI, USA). Polyplexes containing 1.5 μg of mRNA were added to 24-well plates containing HuH-7 cells, seeded at a density of 30,000 cells/well, as described above. Fluorescence observation was performed using a microscope (BX-X800, Keyence, Osaka, Japan) and a 20× objective lens.

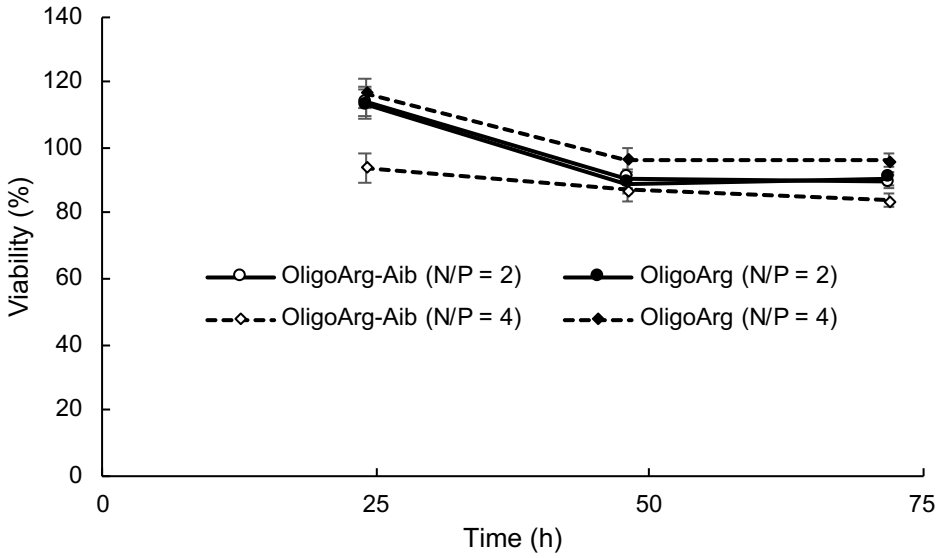
Cell free translation assay

Oligopeptide/mRNA polyplexes were incubated in rabbit reticulocyte lysate (Promega) with final *fLuc* mRNA concentration of 10 μg/mL. After 1 h of incubation, a luciferase assay was performed using Luciferase Assay System and a Lumat^3 LB9508 luminometer.

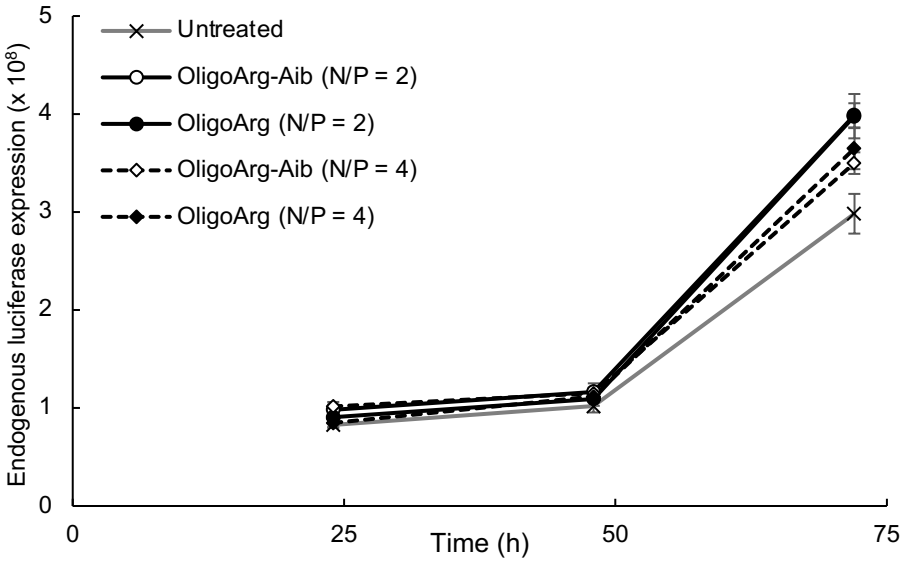
Data presentation and statistical analyses

Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using unpaired two-tailed Student's *t*-tests. * $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

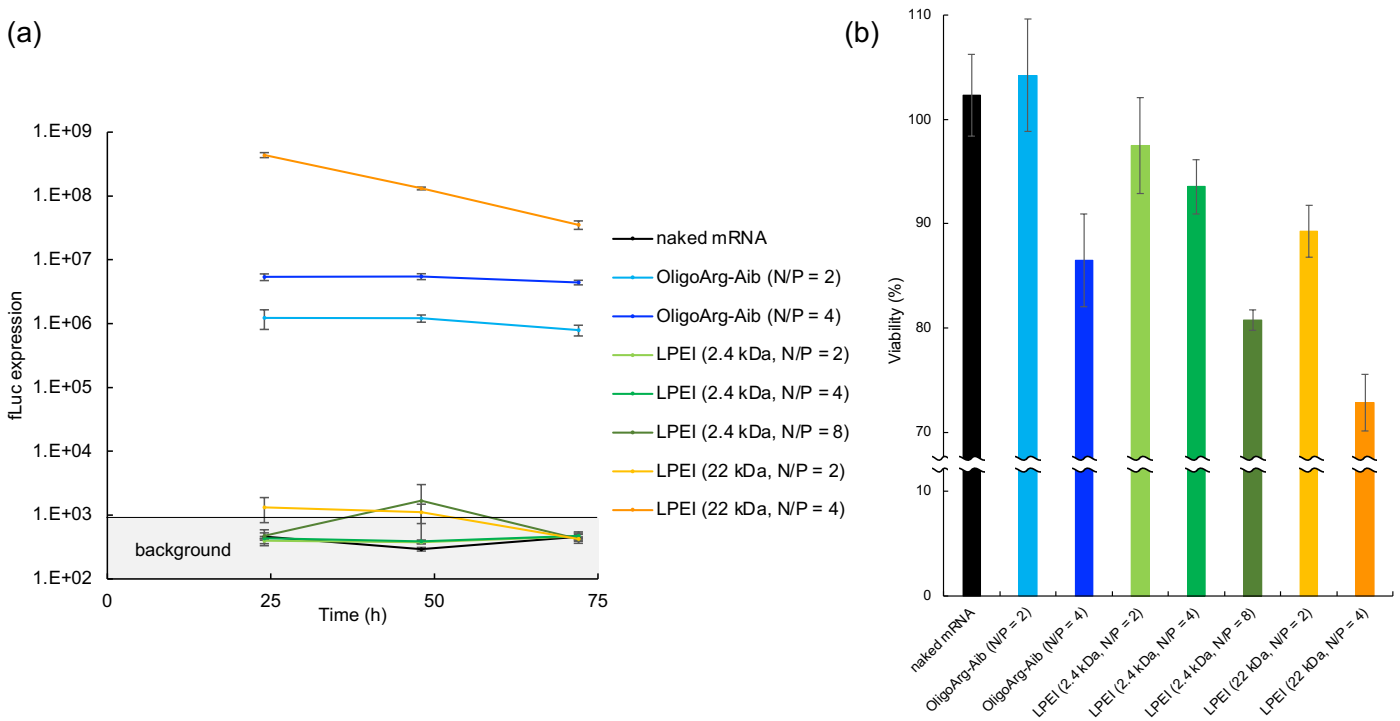
Supplementary Figure



Supplementary Figure 1 Cell viability after mRNA introduction to cultured cells. HuH-7 cells were introduced with *fLuc* mRNA using OligoArg and OligoArg-Aib, at N/P ratio of 2 and 4. $n = 10$.



Supplementary Figure S2 Influence of mRNA introduction using oligopeptides on endogenous protein expression. Using OligoArg and OligoArg-Aib, control mRNA encoding ovalbumin (Trilink, San Diego, CA, USA) was introduced into HuH-7 cells stably transformed to possess luciferase gene in their genome, and the endogenous luciferase expression level was measured to evaluate the influence of oligopeptide/mRNA complexes on endogenous translation machinery. $n = 5$.



Supplementary Figure S3 Introduction of naked mRNA and LPEI/mRNA polyplexes. (a) fLuc expression efficiency after introduction of *fLuc* mRNA to HuH-7 cells. (b) Cytotoxicity 72 h after mRNA introduction. Linear poly(ethylene imine) (LPEI) with two different molecular weights, i.e. 2.4 kDa and 22 kDa was used. Assuming that 50% of amines are protonated at pH 7.4,[2] the number of protonated amines is approximately 28 for 2.4 kDa LPEI and 256 for 22 kDa LPEI, while that in OligoArg-Aib is 10. $n = 6$.

Reference

1. M. Oba, Y. Ito, T. Umeno, T. Kato and M. Tanaka, *ACS Biomaterials Science & Engineering*, 2019, **5**, 5660-5668.

2. J. D. Ziebarth and Y. Wang, *Biomacromolecules*, 2010, **11**, 29-38.