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Supporting Information

A Multi-RNAi Microsponge Platform for Simultaneous Controlled Delivery of Multiple Small Interfering RNAs

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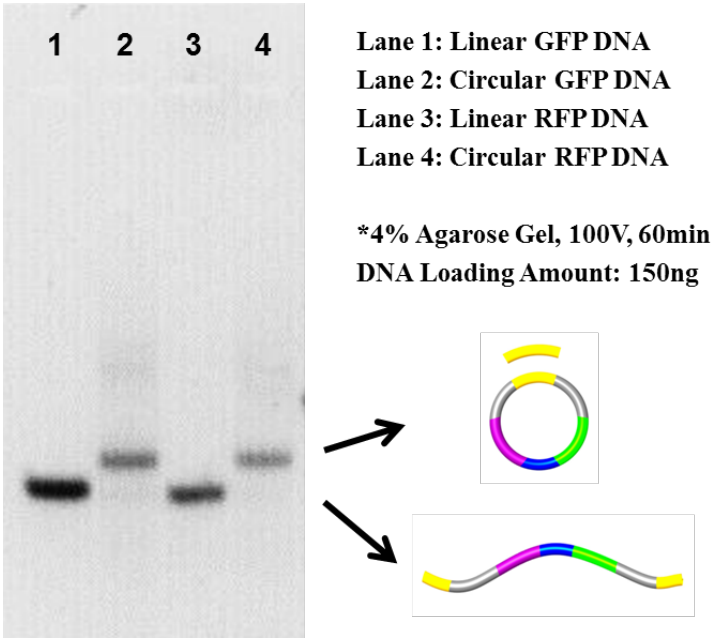
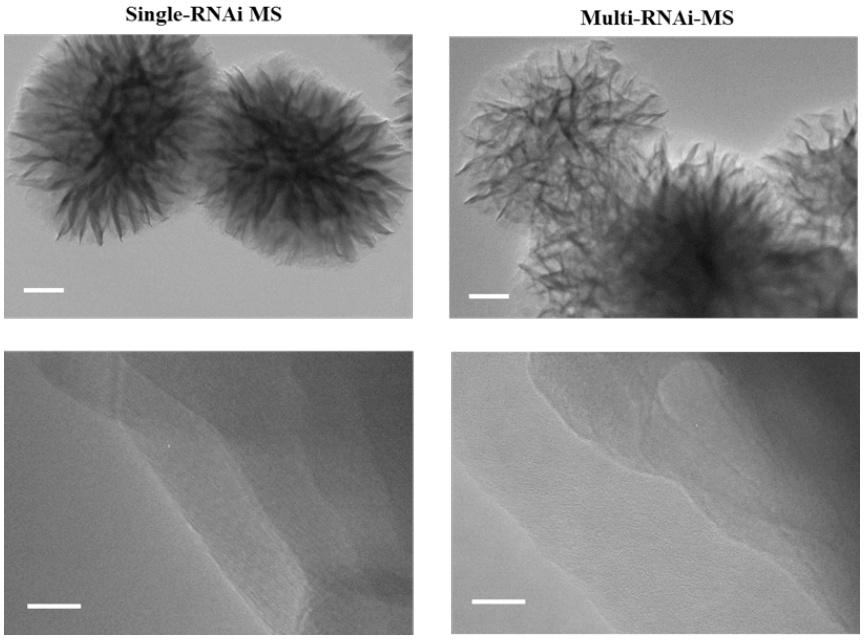
Supplementary Table 1 Oligonucleotide sequences used for the synthesis of Multi-RNAi-MSs.

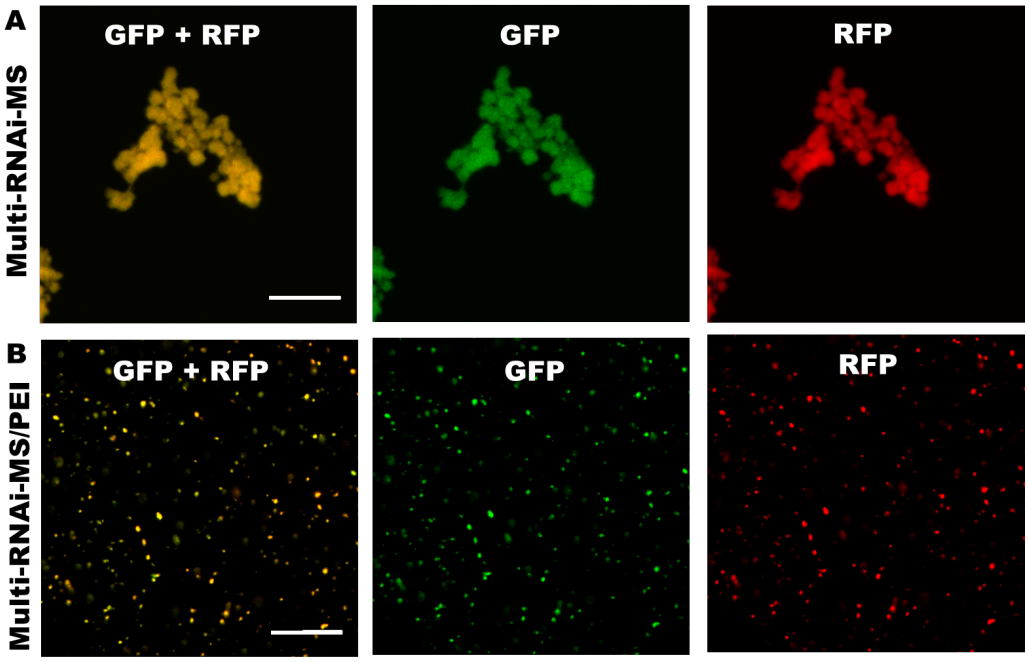
Strand	Sequence
Linear ssDNA (GFP)	5’ Phosphate ATAGTGAGTCGTATTAACGTATTAACGTATACCAACCGGC AAGCTGACCCTGAAGTTCACTTGATGAACTTCAGGGTCA GCTTGCTAGAGGTATTAACGTACATATCCCT 3’
Linear ssDNA (RFP)	5’ Phosphate ATAGTGAGTCGTATTAACGCCACAACGTGTACCAACAAT GTAGATGGACTTGAACTCACTTGAAGAGTTCAAGTCCATC TACATAGAGGCCACAACGTGCATATCCCT-3’
Primer ssDNA	5’-TAATACGACTCACTATAGGGAT-3’
Nucleic Acid Fluorescence Tag (GFP)	5’- FITC TATTAACGTA -3’
Nucleic Acid Fluorescence Tag (RFP)	5’- Cy5 CCACAACGTG -3’



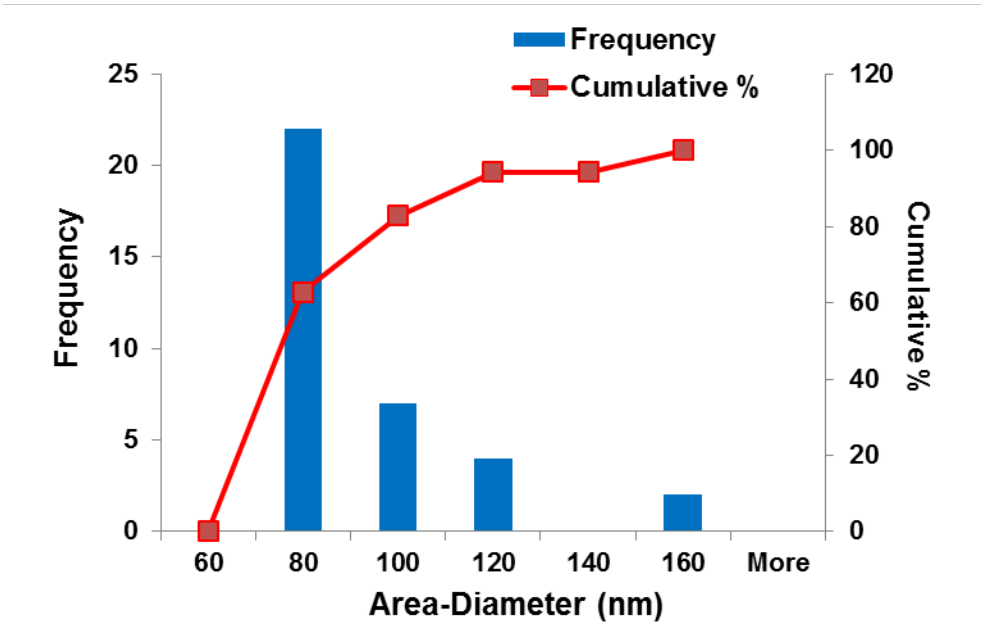
Supplementary Figure 1 Synthesis of various circular DNA templates.
Electrophoretic mobility image of multiple types of linear and circular DNA templates used to synthesize Multi-RNA-MSs. Bandshifts were observed between linear DNAs (Lane 1, 3) and circular DNAs (Lane 2, 4).



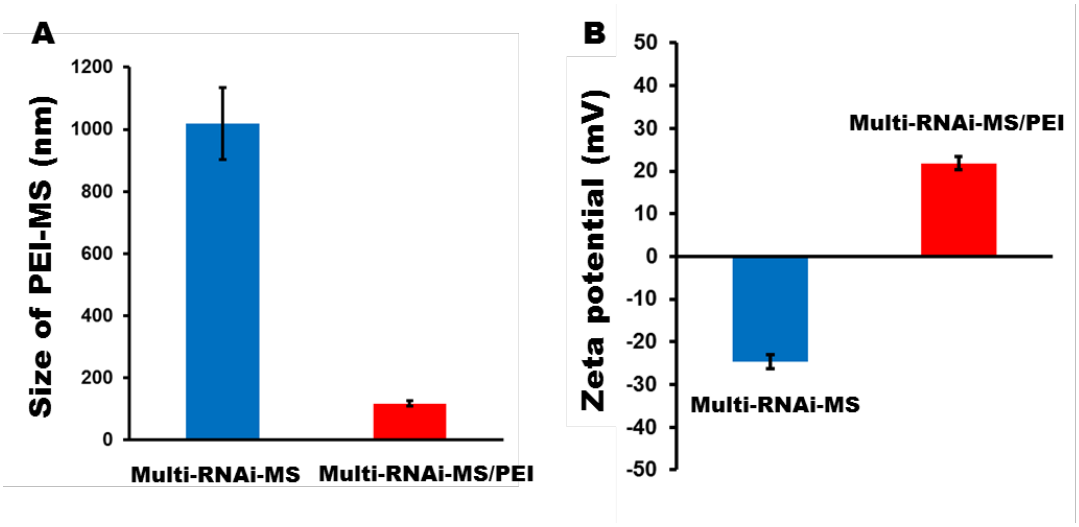
Supplementary Figure 2 Structural characterization of Multi-RNAi-MSs.
TEM images of Single-RNAi-MSs and Multi-RNAi-MSs observed at low magnification (A, B) and at higher magnification (C, D). Scale bars indicate 200 nm in the low magnification and 10 nm in the high magnification images, respectively.



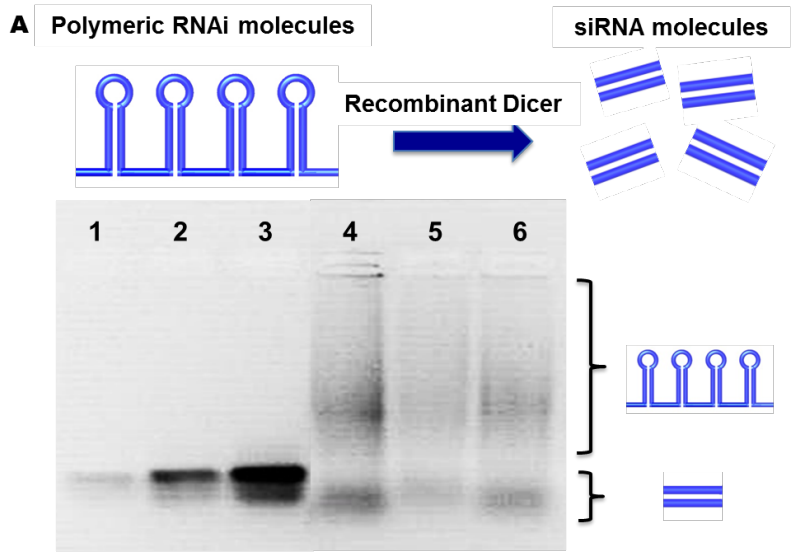
Supplementary Figure 3 Characterization of Multi-RNAi-NPs following condensation. Confocal microscopy images showing co-localization of multiple polymeric RNAi molecules (corresponding to green and red fluorescence respectively) within Multi-RNAi-MSs (A) and Multi-RNAi-NPs (B). Two fluorescent dye-labeled nucleic acid probes (with green and red fluorescence) were used to bind the polymeric RNAi molecules. Green and red fluorescence were both observed in confocal microscopy images, indicating that these condensed structures were still composed of two distinct polymeric RNAi molecules. These results demonstrate that the multiple polymeric RNAi molecular components were well preserved within the condensed particles.



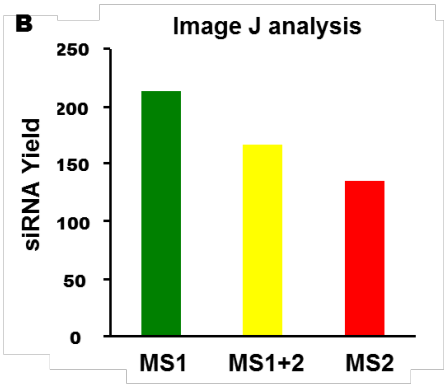
Supplementary Figure 4 Morphology and size distribution of Multi-RNAi-NPs. TEM image of Multi-RNAi-NPs (refer to Fig 4C) processed under particulate analysis using ImageJ1.46r software (NIH, USA), showing a histogram of frequency versus area-diameters of Multi-RNAi-NPs. ImageJ analysis yielded an average particle area-diameter of 102.3 ± 2.2 nm.



Supplementary Figure 5 Characterizations of Multi-RNAi-MSs condensation. A) The average sizes of Multi-RNAi-NPs before and after the condensing process as measured by DLS. B) Surface charge changes of Multi-RNAi-NPs as measured by zeta potential. The average hydrodynamic diameters of Multi-RNAi-MSs after condensation decreased significantly to 117.2 nm ± 8.5 from the original microsponge size of approximately 1019.2 nm ± 115.8). The particle surface charge changed from −24.6 mV ± 1.1 (Multi-RNAi-MSs) to +21.9 mV ± 1.5 (Multi-RNAi-MSs/PEI).

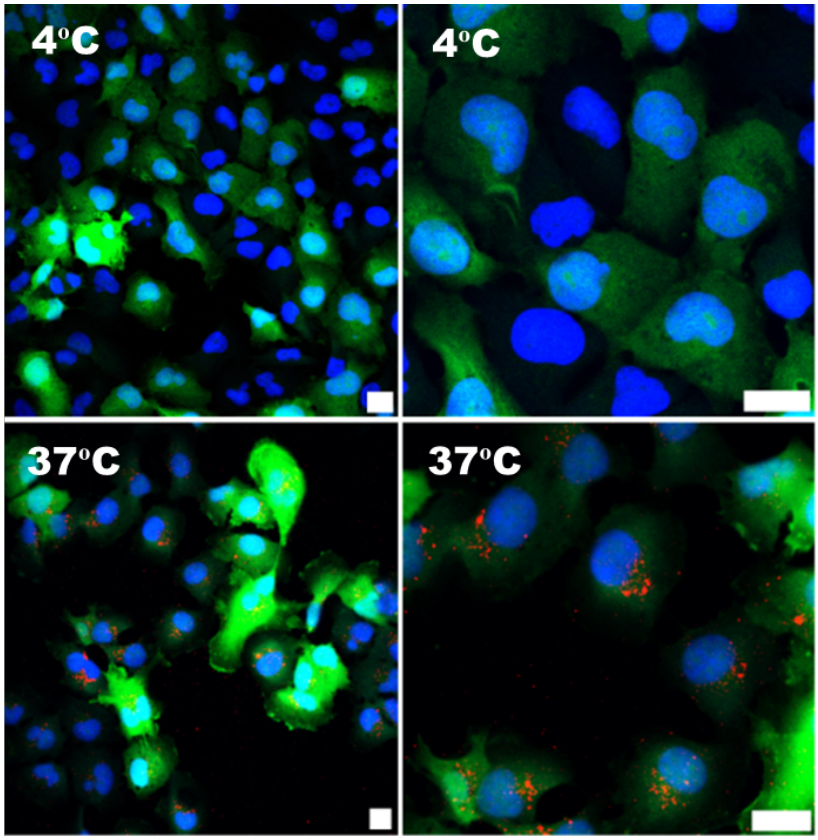


Lane 1: siRNA control (125 ng)
Lane 2: siRNA control (250 ng)
Lane 3: siRNA control (500 ng)
Lane 4: siRNA from Single-RNAi-MS1 after Dicer treatment
Lane 5: siRNA from Single-RNAi-MS2 after Dicer treatment
Lane 6: siRNA from Multi-RNAi-MS1+2 after Dicer treatment

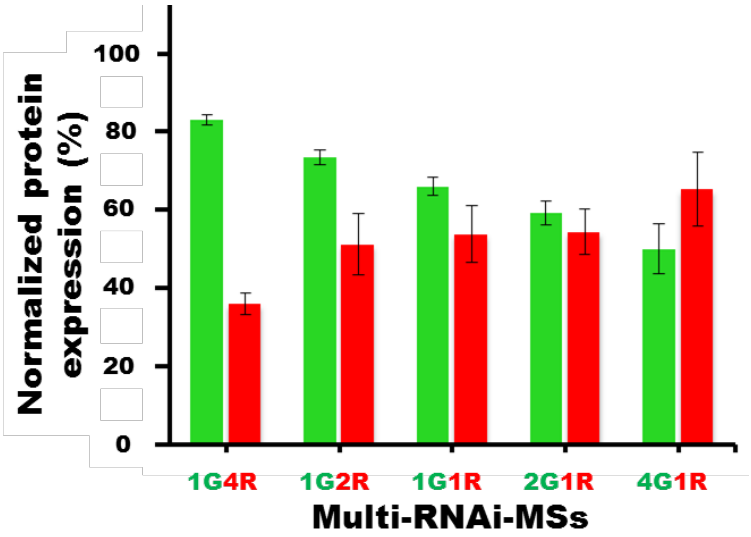


Supplementary Figure 6. Biological function study of multiple polymeric RNAi molecules from Multi-RNAi-MSs. To confirm whether the multiple polymeric RNAi molecules undergo enzymatic processing, Single-RNAi-MSs and Multi-RNAi-MSs were incubated with recombinant Dicer (0.1 unit/μl), and the generation of siRNA products was evaluated by gel electrophoresis and ImageJ analysis. Gel electrophoresis image of polymeric RNAi molecules from Single-RNAi-MSs and Multi-RNAi-MSs after recombinant Dicer treatment (1 unit/μl) for 24 h were compared. Our gel electrophoresis results indicated that the polymeric RNAi molecules from Multi-RNAi-MSs produced similar amounts of siRNAs as those produced by the analogous Single-RNAi-MSs. Careful analysis using ImageJ software also suggested that in the presence of recombinant Dicer, the Multi-RNAi-MSs (containing a 1:1 ratio of two polymeric RNAi molecules) produced an estimated 167 ng of siRNA, indicating that 33% of the cleavable multiple polymeric RNAi molecules was actually diced to individual siRNAs, which

yields an estimated 213 and 135 ng of siRNA produced by digestion of Single-RNAi-MS type 1 and Single-RNAi-MS type 2, respectively. These results demonstrate that polymeric RNAi molecules from Multi-RNAi-MSs can undergo efficient enzymatic digestion by recombinant Dicer and can produce comparable amounts of siRNA compared to Single-RNAi-MSs.



Supplementary Figure 7 Intracellular delivery of Multi-RNAi-NPs. Confocal microscopy images of HeLa cells treated with Multi-RNAi-NPs at 4 and 37°C. Multi-RNAi-NPs were pre-labeled with Cy5-dUTP (corresponding to red color) during a RCT reaction. The actin cytoskeleton was stained green with phalloidin, and the nucleus was stained blue with DAPI. Scale bars: 10 μm.



Supplementary Figure 8 *In vitro* evaluation of Multi-RNAi-NPs in terms of gene silencing. The full range of formulated Multi-RNAi-NPs (i.e., 4G1R, 2G1R, 1G1R, 1G2R and 1G4R) at a concentration of 2 nM was evaluated for knockdown of the target mRNAs. With a pre-defined combination ratio of 4:1, 2:1, 1:1, 1:2, 1:4, two target proteins expression levels were varied and silenced in a ratio-dependent manner respectively.

Methods

Synthesis of a circular DNA template library and Multi-RNAi-MSs.

A variety of circular DNAs templates used for generating Multi-RNAi-MSs were designed from linear ssDNAs, which encoded sequences complementary to the sense and antisense target regions, molecular probe recognition regions, and two primer hybridization sites. Hybridization with a ssDNA primer (22 bases long) and the additional ligation of the hybridized circular form by T4 DNA ligase (1 unit/μl) were performed as described previously.[5] To construct different types of polymeric RNAi molecules incorporated into Multi-RNAi-MSs, pre-selected circular DNA templates were mixed together at differing ratios (4:1, 2:1, 1:1, 1:2, and 1:4) in preparation for RCT. The single-tube reaction mixtures contained reaction buffer (8 mM Tris-HCl [pH 7.5], 0.4 mM spermidine, 1.2 mM MgCl2, and 2 mM dithiothreitol), circular DNA templates (300 nM), T7 RNA polymerase (5 unit/μl), and ribonucleotide triphosphates (8 mM), and were performed at 37°C for 21 h. Polymeric forms of multiple RNAi molecules were simultaneously produced from each circular DNA template, then self-assembled and incorporated into microsponge structures together to construct Multi-RNAi-MSs. A short sonication was used to disperse the Multi-RNAi-MSs, and centrifugal purification (12,000 rpm, 3 min) was used to remove RCT reagents. Fluorescent molecular probes

(Cy5 and FITC) were employed by hybridization with molecular recognition sites in the generated polymeric RNAi molecules to produce fluorescent, color-coded Multi-RNAi-MSs.

Gel electrophoresis

The size, inherent stability and biological functions of polymeric RNAs released from the Multi-RNAi-MSs were characterized via gel electrophoresis. Agarose gels were prepared by mixing agarose (2–4%) with TB buffer and microwaving the resultant mixture for 1 min, followed by the addition of GelRed^{TM} gel stain before the gels solidified. Solidified gels was transferred to an electrophoresis apparatus filled with TB buffer. Gel loading dye containing bromophenol blue (New England Biolabs, Ipswich, MA) was added to each RNA sample following the manufacturer's protocol before being loaded into a well. Gel electrophoresis was conducted at 70–80(V/cm) for 60–70 min, and gel images were visualized using a UV transilluminator (Bio-Rad, Hercules, CA).

Biological function evaluation of the multiple polymeric RNAi molecules from Multi-RNAi-MSs

The biological function, especially that of the enzymatic cleavage by recombinant Dicer, of polymeric RNAs was confirmed by measuring the band intensities of siRNAs produced by polymeric RNAs generated by MS 1, MS 2, and MS 1+2. Five hundred nanograms of MS 1, MS 2, and MS 1+2 were treated with 1.5 units of recombinant Dicer (Genlantis, San Diego, CA) in 15 μl of reaction solution, which included 1 mM adenosine triphosphate, 5 mM $MgCl_2$, and 40% (v/v) Dicer reaction buffer. Samples were incubated overnight and were then inhibited by adding Dicer Stop Solution (Genlantis, San Diego, California). Samples were loaded into 2% agarose gel with 125 ng, 250 ng, and 500 ng of siRNA control as reference. Gel electrophoresis was conducted at 70V for 70 min and the gel image was further processed using densitometric analysis via ImageJ software 1.44p (U.S. National Institute of Health, Bethesda, Maryland) wherein the generated siRNA yield was quantitatively determined and compared by following the previously published protocols.[1]

***In vitro* knockdown experiments and cell viability assays**

GFP-RFP-HeLa/MDA-MB-231 cells were used in *in vitro* knockdown evaluation and cell viability assay. Cells were maintained in growth media consisting of Minimum Essential Media-Alpha (MEM-R), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Stable both GFP and RFP-overexpressing cells were prepared using lentiviral vectors according to the manufacturer's protocols (Cell Biolabs, San Diego, CA). For GFP and RFP silencing evaluation, the cells were first seeded in a 96-well plate at 70% confluence and incubated overnight. The cells were treated with the Multi-RNAi-MSs samples at varied concentrations per well. Two days post transfection, the cells were

harvested with trypsin and the cell-associated GFP/RFP fluorescence was measured by flow cytometry (GFP: Ex 480 nm/Em 530 nm, RFP: Ex 555 nm/Em 584 nm) using a BD LSRFortessa flow cytometer coupled with a high-throughput system for the 96-well plate format (BD biosciences, San Jose, CA). Transfections were conducted in triplicate. The level of GFP and RFP silencing (%) from each sample was obtained by comparing the fluorescence intensity value. The cell viability assays were evaluated using CCK-8 kit (Dojindo Molecular Technologies, Rockville, MD) following the manufacturer's protocols and using a microplate reader (Tecan Infinite 200 PRO).

References

[1] J. B. Lee, J. Hong, D. K. Bonner, Z. Poon, P. T. Hammond, *Nature Materials* **2012**, *11*, 316-322.