Supporting Information

Multivalent Aptamer-RNA based Fluorescent Probes for Carrier-Free Detection of Cellular MicroRNA-34a in Mucin1-Expressing Cancer Cells

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Experimental details

1. Materials

Mature miR-34a, RNA complementary to miR-34a (c_miR-34a), non-complementary random RNA (nc_RNA), c_miR-34a with an oligoA overhang (10-base pairs) at both 5′- and 3′-terminal ends (dA_c_miR-34a), c_miR-34a with an oligoA overhang (20-base pairs) at the 5′-end (monovalent dA_c_miR-34a), Cyanine 5.5 (Cy 5.5)-modified RNAs (a1), Black Hole Quencher® (BHQ)-2 modified RNAs (a2), mucin1 (MUC1) aptamer, miR-200c, and miR-143 were purchased from Bioneer Co. (Daejeon, Republic of Korea). The appropriate quencher-fluorophore combination was selected according to a previous study [1]. Locked nucleic acid (LNA)-modified RNAs, 34a2 with BHQ-2 at the 3′-end and 34a1 with Cy 5.5 at 5′-end were obtained from Eurogentec (Seraing, Belgium). The dA_c_miR-34a that was thiol-modified at both terminals was purchased from Integrated DNA Technologies (Coralville, IA, USA). Nucleic acid sequences of all oligos are given in Table s1. Diethylpyrocarbonate (DEPC) and glutathione were purchased from Sigma (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium and penicillin/streptomycin (P/S) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals and reagents were of analytical grade.

2. Characterization of LNA-based probes

Two types of probes, LNA-modified RNA probes (LNA probe; c_miR-34a/34a1/34a2) and RNA-based probes (RNA probe; c_miR-34a/a1/a2), were prepared via complementary hybridization. To prepare the LNA probe, c_miR-34a (0. 125 nmol) was annealed to 34a1 (0.125 nmol) and 34a2 (0.375 nmol) for 20 min at room temperature (RT). To prepare the RNA probe, c_miR-34a (0. 125 nmol) in PBS solution was hybridized with complementary a1 (0.125 nmol) and a2 (0.375 nmol) probes for 20 min at room temperature (RT). Each probe in PBS solution was incubated at different temperatures, ranging from 25 °C to 80 °C, for 15 min. After mixing each sample with dilution solution (100 mM Tris-HCl, 1 mM $MgCl_2$, 1% Tx-100, pH 8.0), fluorescence intensity (FI) were measured using a fluorospectrophotometer (Molecular Device, Sunnyvale, CA, USA) at excitation and emission wavelengths of 673 nm and 707 nm, respectively. The FI of each probe at different temperatures was normalized to the FI of the Cy 5.5-labeled oligonucleotides.

LNA probes (0.1 nmol) and RNA probes (0.1 nmol) were incubated with different concentrations of miR-34a and nc-RNA (0, 0.1, and 1 nmol) for 1 h at 37°C, respectively. The fluorescence signal of each sample in PBS solution with 1% Tx-100 was visualized and quantitatively analyzed using an IVIS® imaging system (Caliper Life Sciences Lumina II, Hopkinton, MA, USA). As a control, we used a probe without a quencher (a2 or 34a2). To determine the detection limit of LNA probes, the LNA-based probe (150 pmol) was incubated with different concentrations of miR-34a (0, 100 zM, 10 aM, 1 fM, 100 fM, 10 pM, 1 nM, and 100 nM) in PBS solution for 1 h at 37 °C. Then, each sample was diluted in PBS solution containing 1% Tx-100. After the diluted samples were loaded into 96-well plates, they were visualized and quantitatively analyzed using the IVIS® imaging system.

3. Preparation of CF-probes

Multimeric c_miR-34a conjugates were prepared according to our previous study, with minor modifications [2]. Briefly, the thiol-modified c_miR-34a (dA_c_miR-34a; 5 nmol) was treated with DTT solution (1 M) for deprotection overnight and then purified using a desalting column (Pierce, Rockford, IL, USA). The resulting solution was allowed to react with dithiobismaleimidoethane (DTME, 5 nmol) overnight. The multimeric c_miR-34a conjugates (0.1 nmol) were annealed with complementary miR-34a (0.1 nmol) and 34a1 (0.1 nmol) for 20 min, respectively, and then electrophoresed on 15% polyacrylamide gels. For cleavage of disulfide bonds within multimeric c_miR-34a conjugates, the conjugates were incubated with glutathione (GSH) solution (10 mM) for different time periods (0.5, 2, and 5 h). After electrophoresis for 50 min at 180 V, the fluorescence signals in gels were visualized using an IVIS® imaging system. To prepare CF-probes, multimeric c_miR-34a conjugates (0.1 nmol) were hybridized with the MUC1 aptamer (0.1 nmol), 34a1 (0.1 nmol), and 34a2 (0.3 nmol), by first melting (at 95 °C for 5 min) and subsequently cooling the mixtures to 25 °C for 2 h in an Biometra thermocycler machine[3]. As a control, the LNA probe (0.1 nmol) was also annealed with the MUC1 aptamer (0.1 nmol), 34a1 (0.1 nmol), and 34a2 (0.3 nmol) under the same conditions to prepare an aptamer-labeled LNA (Apt-LNA) probe.

4. Intracellular uptake of CF-probes

MCF-7 cells (a human breast cancer cell line) were maintained in RPMI supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. MCF-7 cells were plated in 6-well plates at a density of 6×10^5 cells/well for 24 h prior to transfection. The CF-probe without a quencher (Q) and the Apt-LNA probe without Q were used to treat cells at a Cy 5.5 concentration of 200 pmol for 4 h. After incubation with each probe, cells were washed with PBS three times. The FI within cells were analyzed by FACS (Becton Dickinson, Franklin Lakes, NJ, USA) after fixation of cells with 3.7% formaldehyde in PBS solution.

5. Detection of miR-34a in cancer cells

T47D cells (a human breast cancer cell line) were maintained in RPMI supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. T47D cells were plated in 6-well plates at a density of 1.2×10^6 cells/well for 24 h prior to transfection. CF-probes without and with Q were used to treat cells at a Cy 5.5 concentration of 250 pmol for 4 h. After replacing the medium with fresh RPMI, miR-34a at a concentration of 250 nM was transfected into the cells by using Lipofectamine at a Lipofectamine/RNA weight ratio of 0.5. Cells were washed with PBS solution three times. After detachment and resuspension in PBS, live cells were visualized using the IVIS® imaging system. The FI within cells treated with probes were also analyzed by FACS (Becton Dickinson, Franklin Lakes, CA, USA) after fixation of cells with 3.7% formaldehyde in PBS solution. The percentage of fluorescent cells sorted within a pre-fixed gate region was analyzed as described previously[2].

MCF-7 cells were plated in 4-well chamber slides at a density of 1×10^5 cells/well for 24 h prior to transfection. The CF-probe (125 pmol) was incubated with the cells for 5 h in the absence of carriers. After exchanging the medium for fresh RPMI, four types of different miRNAs (miR-34a, nc-RNA, miR143, and miR200), at a concentration of 250 nM, were transfected with Lipofectamine at a Lipofectamine/RNA weight ratio of 0.5. Cells were washed with PBS solution twice and were fixed with 3.7% formaldehyde in PBS solution. Cells treated with the CF-probe without Q were used as control. The FI within cells were visualized using confocal microscopy (LSM 710, Carl Zeiss, Oberkochen, Germany).

To detect endogenous miR-34a, MUC1-expressing A549 cells (a human lung cancer cell line) were maintained in RPMI supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2[4]. A549 cells were plated in 4-well chamber slides at a density of 1×10^5 cells/well for 24 h prior to transfection. Either CF-probe or CF-probe without Q (125 pmol) was transfected into cells for 4 h. After washing cells with PBS solution and fixation, FI within cells were observed immediately using a confocal microscopy. In addition, cells were incubated for 4 days in fresh RPMI1640 with serum after treatment with CF-probes for 4 h. After incubation, FI within cells were observed using a confocal microscopy. A549 cells were plated in 6-well plates at a density of 8×10^5 cells/well for 24 h prior to transfection. CF-probes without and with Q were used to treat cells at a Cy 5.5 concentration of 250 pmol for 4 h. After further incubation for 0 and 4 days, cells were washed with PBS solution three times. Then, fluorescence intensities in cells were analyzed using an IVIS® imaging system after detachment and resuspension in PBS solution.

6. Biocompatibility of CF-probes

MCF-7 cells were plated in 96-well plates at a density of 5×10^3 cells/well for 24 h prior to transfection. CF-probes were treated to cells at different RNA concentrations (0, 1.75, 3.5, 7, 14 μg/mL) for 5 h without additional reagents. LNA probes were mixed with Lipofectamine at a Lipofectamine/RNA weight ratio of 0.5 to prepare Lipofectamine/LNA probes for cellular uptake as

controls. After treatment each sample, the media containing probes were replaced with fresh culture media. After incubation for 1 and 2 days, cell viability was measured using a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) assay.

References

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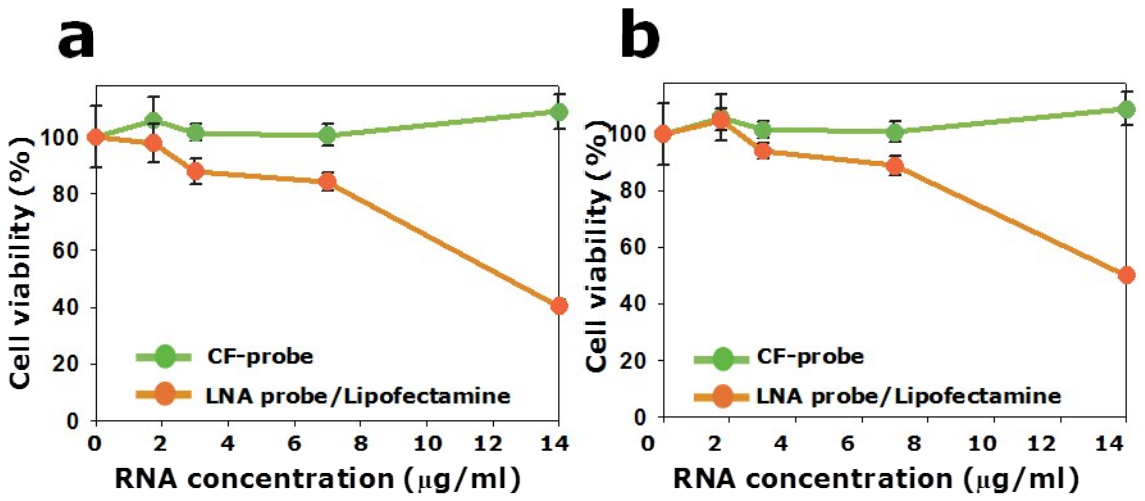
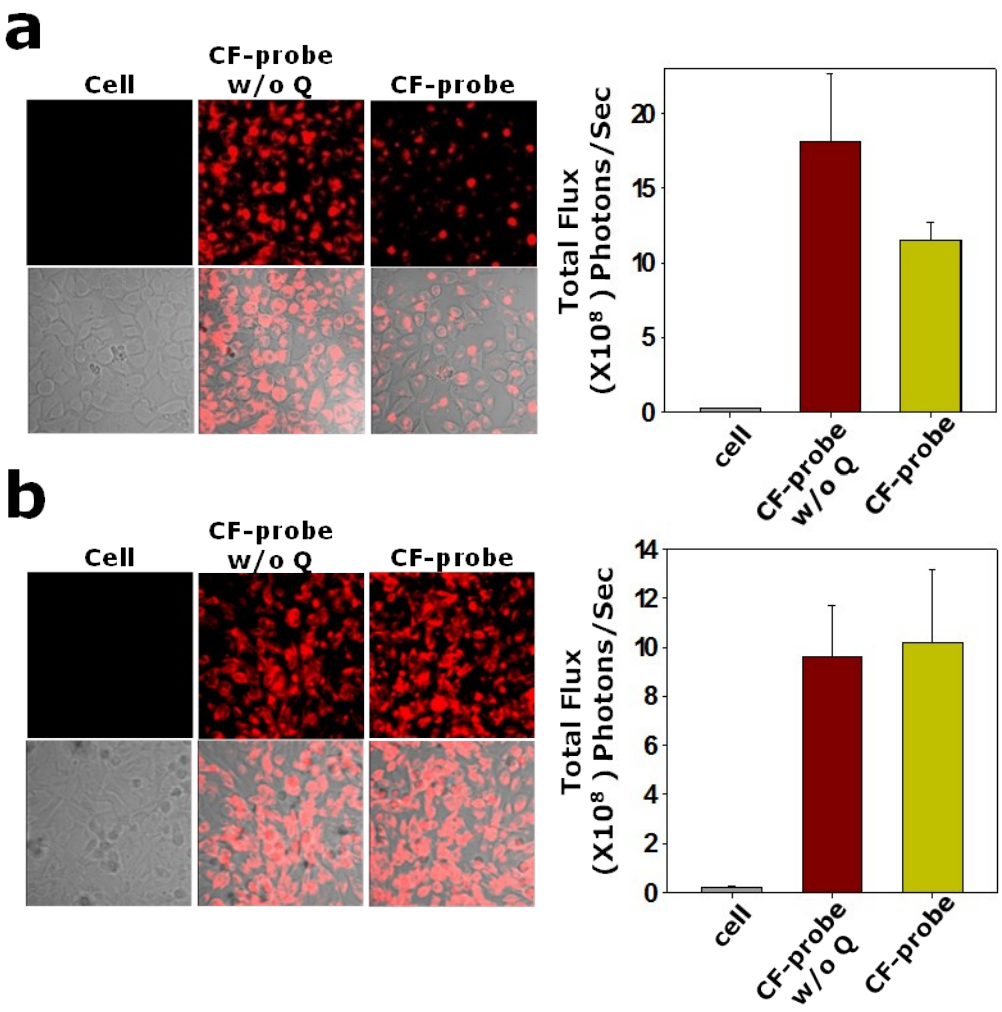
(3) Yoo, H.; Jung, H.; Kim, S. A.; Mok, H. *Chem Commun (Camb)* **2014**, *50*, 6765.
(4) Ferreira, C. S.; Cheung, M. C.; Missailidis, S.; Bisland, S.; Gariepy, J. *Nucleic Acids Res* **2009**, *37*, 866.



Supplementary Figure 1. Cell viability of MCF-7 cells treated with CF probes and LNA probe/Lipofectamine mixtures at different RNA concentrations for 5 h. As a control, cells were treated with LNA probes mixed with a commercially available cationic lipid carrier (Lipofectamine). After (a) 1 day and (b) 2 days of incubation, cell viability was measured using a CCK-8 assay.



Supplementary Figure 2. Monitoring of endogenous miR-34a in A549 cells using (left panel) confocal microscopy and (right panel) IVIS imaging system after treatment with CF-probes to A549 cells for 4 h and further incubation (a) for 0 h and (b) for 4 days, respectively.

Oligo name	Sequence	Length (base pairs)
miR-34a	5′-UGGCAGUGUCUUAGCUGGUUGU-3′	22
c_miR-34a	5′-ACAACCAGCUAAGACACUGCCA-3′	22
dA_c_miR-34a	5′-*AAAAAAAAAA*ACAACCAGCUAAGACACUGCCA*AAAAAAAAAA*-3′	42
monovalent dA_c_miR-34a	5′-*AAAAAAAAAAAAAAAAAAAAAA*CAACCAGCTAAGACACTGCCA-3′	42
Thiol-modified dA_c_miR-34a	5′-HS-*AAAAAAAAAA*ACAACCAGCUAAGACACUGCCA*AAAAAAAAAA*-SH-3′	42
MUC1 Aptamer	5′-*GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTTTTTTTTTT*-3′	45
a1	5′-CY5.5-UAGCUGGUUGU-3′	11
a2	5′-UGGCAGUGUCU-BHQ2-3′	11
34a1	5′-CY5.5-UA**G**CU**G**GUUGU-3′	11
34a2	5′-UG**G**CAGUGUCU-BHQ2-3′	11
nc-RNA	5′-GCUGCACCUGACGCCCUUCTT-3′	21
miR-200c	5′-UAAUACUGCCGGGUAAUGAUGGA -3′	23
miR-143	5′-UGAGAUGAAGCACUGUAGCUC-3′	21

Supplementary Table S1. Sequences of oligonucleotides used in this study. Mature microRNA-34a (miR-34a), RNA complementary to microRNA-34a (c_miR-34a), miR-34a with an oligoA overhang at both terminals (dA_c_miR-34a), c_miR-34a with an oligoA overhang at the 5′-end (monovalent dA_c_miR-34a), cyanine 5.5 (Cy 5.5)-modified RNAs (a1), Black Hole Quencher® (BHQ)-2-modified RNAs (a2), mucin 1 (MUC1) aptamer, RNA complementary to miR-34a (a1, a2); locked nucleic acid (LNA)-modified RNA complementary to miR-34a (34a1, 34a2); non-complementary random RNA (nc_RNA), mature microRNA-200c (miR-200c), and mature microRNA-143 (miR-143). LNA modifications are indicated in bold and underlined text. Deoxyribonucleic acids are indicated in italic font.