Electronic Supporting Information

Fluorescence amplified sensing platform enabling miRNA detection by self-circulation of molecular beacons circuit

Preparation and characterization of molecular beacons (MBs)

We designed 4 types of MBs (MB A, B, C and D; i.e., the MBs circuit), and microRNA-34a (miRNA-34a) and control RNA (Ctrl RNA) were purchased from Bioneer, Inc. (Daejeon, Korea) (sequences shown in Table S1, Supporting information). Among these MBs, only MB A was modified with Cy5 at the 5' end as a donor and black hole quencher 2 (BHQ-2) at the 3' end as an acceptor. Each MB was suspended in duplex buffer (100 mM potassium acetate; 30 mM HEPES, pH 7.5) (IDT, USA) at a concentration of 62.5 ~ 500 nM. After the prepared MBs were annealed by heating for 5 min at 95 °C to form hairpin structures, the system was allowed to cool to room temperature. To confirm the annealing, the fluorescence intensity was measured; after treating miRNA-34a with the MBs, the fluorescence intensity was measured in triplicate using an Infinite M200 PRO instrument (Tecan, Australia) every 10 min for 2 hours at 37 °C (λem: 668 nm, λex: 620 nm). Control miRNA (Ctrl miRNA) was tested in the same manner.[1] We confirmed that the MB A sequence was properly designed to detect miRNA-34a. First, MB A was annealed to form a hairpin loop structure, and its fluorescence intensity was decreased due to strong quenching by fluorescence resonance energy transfer (FRET). Then, we treated miRNA-34a with various concentrations of MB A and measured changes in the fluorescence intensity at 37 °C for 120 min. We also used 15-base-mismatched miRNA-34a (Ctrl miRNA) as a negative control and MB A only, without miRNA target, as a blank control in the same manner (Fig. S3). In the presence of miRNA-34a, the fluorescence signal increased over time, and the intensity at 180 min showed a tendency to increase with increasing MB concentration. However, when the Ctrl miRNA was treated or only the MB was present, MB A was inactivated, and there were almost no changes in the fluorescence signal regardless of the MB concentration. We decided to use MB at 25 pmol based on these results (Fig. S3).

Evaluation of the capability of the MBs circuit for miRNA-34a detection.

Various concentrations of miRNA-34a (2.5 ~ 25 pmol in 10 μL) were treated with the annealed MBs, and the resulting fluorescence intensity was measured every 10 min for 2 hours at 37 °C. As comparison groups, Ctrl miRNA instead of miRNA-34a was treated, or only MB A was used instead of the MBs circuit. The fluorescence intensities were measured in triplicate using an Infinite M200 PRO instrument (Tecan, Australia) at an emission and excitation wavelength of 668 nm and 620 nm, respectively.

Synthesis and characterization of mPEG-PEI copolymer.

We purchased methoxy polyethylene glycol (mPEG) (MW: 5,000 Da), hexane-1,6-diiocyanate (HDMI), branched polyethylene imine (bPEI) (MW: 25,000 Da) and chloroform ($CHCl_3$) from Sigma-Aldrich. mPEG-PEI copolymer was prepared using a two-step procedure (Fig. S6a).[2,3] First, mPEG (MW: 5,000 Da) was activated with hexane-1,6-diiocyanate to form PEG-HDMI. mPEG (10 g) dissolved in $CHCl_3$ (20 mL) was then added HDMI (~ 200 × molar excess) (40 mL) in a 100-mL round-bottom flask and refluxed under nitrogen for 16 hours to concentrate the mPEG-HDMI mixture. The mPEG was dissolved in a minimal amount of $CHCl_3$ and then precipitated into hexanes (500 mL). Precipitated PEG-HDMI was then subjected to vacuum filtration using fine filter paper. These re-precipitation and washing steps were repeated three times before the polymer was isolated to remove excess HDMI. After drying the material for 48-72 hours, we obtained PEG-HDMI, as confirmed by Fourier-transform infrared (FTIR) spectroscopy. Then, bPEI (MW 25,000) (1 g) and PEG-HDMI (2.06 g) were mixed in 100 mL of $CHCl_3$ and refluxed under N_2. After 16 hours, this mixture was concentrated using an evaporator, precipitated into Et_2O, dried under high vacuum and stored at -20 °C in sealed vials under N_2. After completion of the reaction, we verified the chemical bonds of PEG-PEI by proton nuclear magnetic resonance (^{1}H-NMR) and FTIR spectroscopy.

As shown in Fig. S6b, they commonly showed peaks corresponding to the C-H stretching and C-O ether stretching of mPEG at 2921 cm^{-1} and 1106 cm^{-1}, respectively. mPEG-HDMI was confirmed by the characteristic bands at 2274 cm^{-1} (O=C=N isocyanate stretching) and 1724 cm^{-1} (C=O urethane stretching). After the final coupling of mPEG-HDMI with PEI to form synthesized mPEG-PEI, only characteristic bands for amide bonds at 3300 cm^{-1} (N-H stretching) and 1600 cm^{-1} (N-H banding) were observed (Fig. S6b). In addition, we identified the ^{1}H-NMR peaks of mPEG-PEI corresponding to mPEG (-CH_2CH_2O-) and PEI (-CH_2CH_2NH-) (Fig. S6c).

Viability of MDA-MB-231 cells treated with mPEG-PEI.

We evaluated the viability of MDA-MB-231 breast cancer cells treated with mPEG-PEI by measuring the inhibition of cell growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDA-MB-231 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 5 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (P/S). Cells (1×10^4 cells/well) were plated in a 96-well plate at 37 °C, allowed to attach overnight, and then incubated with various concentrations of mPEG-PEI for 24 hours. After incubation, the cells were washed with phosphate-buffered saline (PBS), and the MTT assay was performed, in which yellow tetrazolium salt was reduced to purple formazan crystals in metabolically active cells. The relative percentage of cell viability was determined as the ratio of the formazan intensity in viable cells treated with mPEG-PEI to that in nontreated (control) cells. Cell viability was normalized to that of nontreated cells, which were considered to be 100 % viable. It has been reported that in the form of mPEG-PEI, mPEG lowers the cytotoxicity of PEI by shielding its surface charge through PEGylation; additionally, mPEG-PEI has been shown to result in greater than 80 % cell viability without any inhibitory effects on cell growth at high concentrations (< 10 μg/mL) compared with no treatment (NT; 0 μg/mL) (Fig. S7).

Formulation of MBs circuit/mPEG-PEI complexes.

We formulated MB/mPEG-PEI complexes for the stable transfer of the MBs circuit to cells. First, to determine the optimal miRNA-34a loading condition, MBs (1 μg) were mixed with mPEG-PEI at different concentrations for 30 min at room temperature (RT) and then analyzed by gel retardation assay. Each complex was mixed with DNA loading dye, loaded onto 3.0 wt % agarose gel and electrophoresed in Tris-borate-EDTA (TBE) buffer at 100 V for 40 min. Exposed MBs were visualized under UV light using a ChemiDoc™ Gel documentation system. A total of 70 ng of MBs (17.5 ng of each MB) and mPEG-PEI (70 μg) were added to Opti-MEM (Gibco) and mixed at RT for 30 min to produce MB/mPEG-PEI complexes. As a control, MB A/mPEG-PEI complexes were prepared using 17.5 ng of only MB A and mPEG-PEI (17.5 μg) in the same manner. MB A and mPEG-PEI were sufficiently condensed via electrostatic interactions in TE buffer and formed MB A/mPEG-PEI complexes; these complexes were electrophoresed on a 3 % agarose gel at various mPEG-PEI:MB A weight ratios. The band corresponding to the naked MBs represents the presence of only MBs (MB:mPEG-PEI=1:0),

and the intensity of this band gradually decreased as the weight ratio of mPEG-PEI increased (Fig. S8). Notably, retardation of the MBs occurred at weight ratios greater than 0.75, confirming that mPEG-PEI efficiently condensed the MBs and would prevent them from being exposed to potential enzymatic or physical degradation during blood circulation. The average size of MBs circuit/mPEG-PEI complexes was analyzed by laser scattering (ELS-Z, Otsuka Electronics) that was 159.3 ± 15.2 nm.

***In vitro* imaging of miRNA-34a using MBs circuit/mPEG-PEI complexes.**

MDA-MB-231 cells were seeded on μ-Slide 8-well slides (chambered coverslips) (10^5 cells/well) and incubated overnight. The cell media was removed from the prepared slides, and the cells were then treated with the MBs circuit/mPEG-PEI and MB A/mPEG-PEI complexes at 37 ℃ in a 5 % CO_2 atmosphere for 4, 8, 16 and 24 hours. As a control, the cells received no treatment (NT); 200 μL of Opti-MEM was added to the wells and incubated as described. After incubation, the MDA-MB-231 cells were fixed with Fixation/Permeabilization Solution (BD Cytofix/Cytoperm) and then stained with Hoechst 33342 (Thermo) to visualize the cell nuclei. The cells were observed using a DeltaVision microscope system (GE); then, the cells were detached from the slide with TrypLE Express (Gibco) and resuspended in 1 % FBS. The intensity of Cy5 fluorescence after miRNA detection by the MBs was analyzed using a BD FACSAria flow cytometer.

***In vivo* optical imaging of miRNA-34a detection by MBs circuit.**

All animal experiments were conducted with the approval of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. To detect miRNA-34a in vivo via optical imaging, we developed an orthotopic breast cancer tumor model by injecting MDA-MB-231 cells (10^7 cells suspended in 200 μL of PBS per animal) into the mammary fat pad of female BALB/c-nude mice aged 4-5 weeks.[1,4] After cancer cell implantation, we monitored the tumor formation for 2-3 weeks, and optical imaging was then performed. Optical tomographic images were obtained using an IVIS® Imaging system (Xenogen, USA) with a Cy5 channel (Excitation filter: 640 nm and Emission filter: 680 nm).[5-8] Imaging was performed for 1 hour after injection of the MBs circuit/mPEG-PEI and MB A/mPEG-PEI complexes, and the obtained images were assessed using an image analysis program.

***Ex vivo* optical imaging.**

After optical image acquisition, the mice were sacrificed, and the selected tissues (brain, liver, kidney, spleen, heart, muscle and tumor) were excised and kept in saline. The relative photon counts of the excised organs were measured using the IVIS® imaging system (Xenogen, USA) (Excitation filter: 640 nm and Emission filter: 680 nm). In addition, the extracted tumor tissues were cryo-sectioned and stained with Hoechst® 33342 (Thermo). The tissue sections were then analyzed by confocal laser scanning microscopy (CLSM; LSM 700, Carl Zeiss).[9, 10]

1. E. Kim, J. Yang, J. Park, S. Kim, N. H. Kim, J. I. Yook, J.-S. Suh, S. Haam and Y.-M. Huh, *ACS Nano*, 2012, **6**, 8525-8535.
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Name	Sequence (5' – 3')	Base pairs
miRNA-34a	UGGC AGUG UCUU AGCU GGUU GU	22
MB A	Cy5-GTGT CTTA GCTG GTTG GGAA AGTG GGCA GTGT CAAC CAGC TA AG ACAC TGCC-BHQ-2	52
MB B	GTTG GGAA AGTG GGCA GTGT CTTA GCTG GTTG ACAC TGCC CACT TTCC CAAC CAGC	56
MB C	CACT TTCC CAAC CCGG TAAG AAGG AGTG GGCA GTGT CGGA CCGG GTTG GGAA	52
MB D	CCGG TAAG ACAC TGCC CACT TTCC CTTA CCGG GTTG GGAA AGTG GGCA GTGT CTTA GCTG GTTG	64
Ctrl miRNA	UUCG UCAA UGGG AAGA GCUC GU	22

Table S1. The sequence of miRNA-34a, control (Ctrl) miRNA and each molecular beacon (MB) (A, B, C and D).

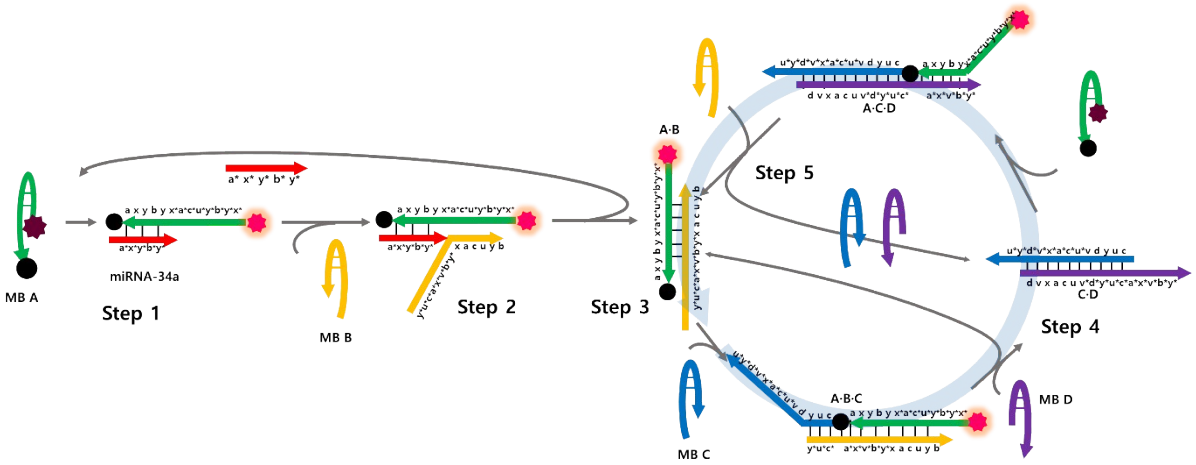

Figure S1. Illustration of enzyme-free fluorescence signal amplification process through the self-circulation reaction of molecular beacons (MBs) circuit (MBs; MB A, B, C, and D) for the highly sensitive detection of miRNA-34a. (Red: miRNA-34a, green: MB A, yellow: MB B, blue MB C, purple: MB D).

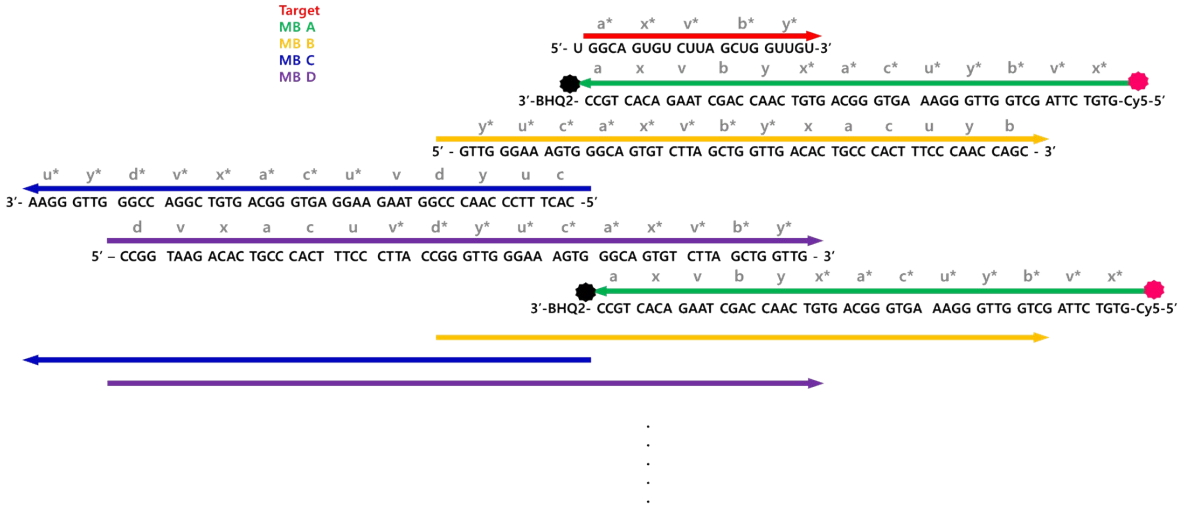

Figure S2. Illustration of the self-circulation reaction process of MBs circuit through sequential opening by hybridization reaction of MBs (Red: miRNA-34a, green: MB A, yellow: MB B, blue MB C, purple: MB D).

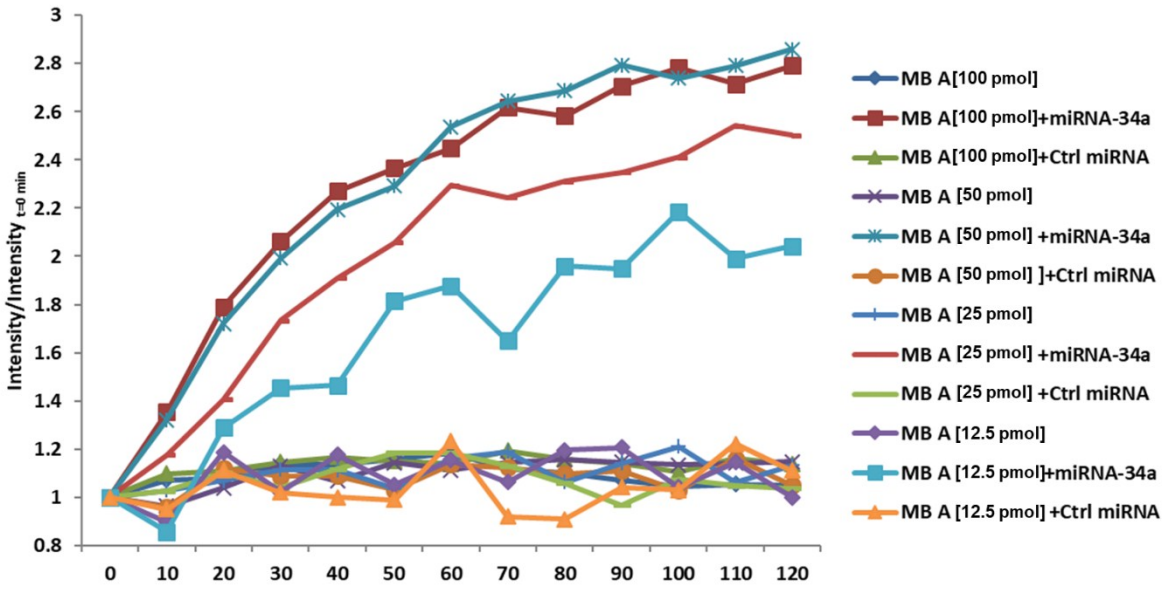

Figure S3. Changes in the fluorescence intensity (Intensity/Intensity$_{t=0min}$) after miRNA-34a detection at various concentrations of MB A alone for 2 hours (120 min). Ctrl miRNA was used as a control.

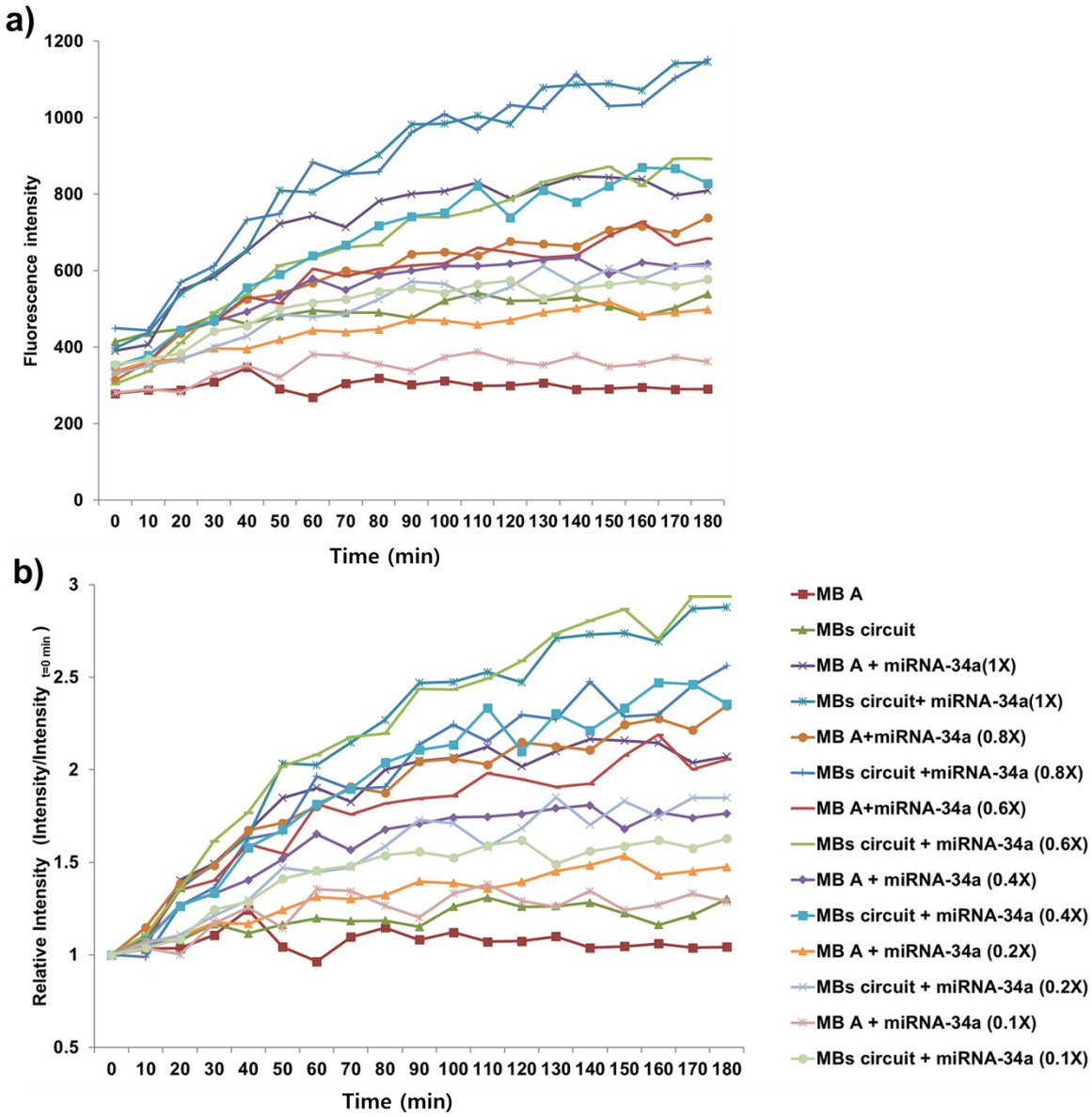

Figure S4. (a) Fluorescence intensity of MB A and other MBs circuit and (b) their intensity ratios ($Intensity_{t=n\ min}/Intensity_{t=0\ min}$) over 3 hours (180 min) of detecting miRNA-34a at various concentrations (1 ×: 25 pmol, 0.8 ×: 20 pmol, 0.6 ×: 15 pmol, 0.4 ×: 10 pmol, 0.2 ×: 5 pmol, 0.1 ×: 2.5 pmol). Ctrl miRNA was used as a control.

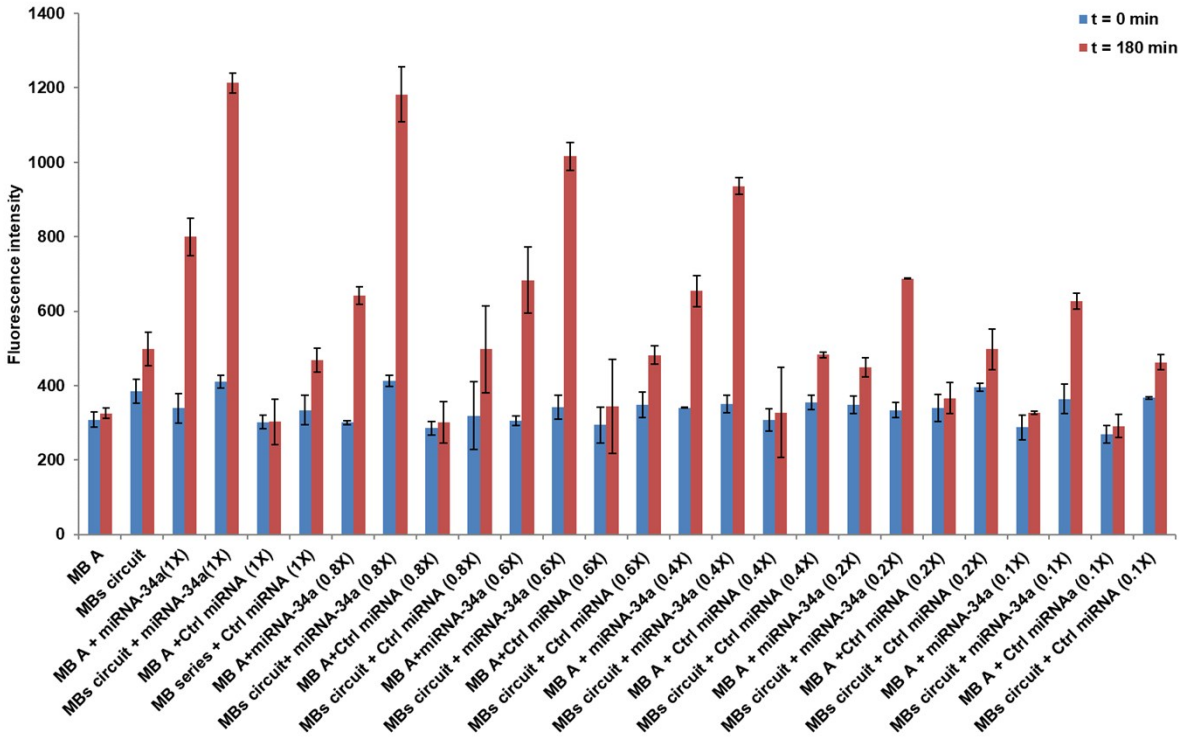

Figure S5. Fluorescence intensity of MB A and MBs circuit before (t=0 min) and after (t=180 min) combination with miRNA-34a at various concentrations (1 ×: 25 pmol, 0.8 ×: 20 pmol, 0.6 ×: 15 pmol, 0.4 ×: 10 pmol, 0.2 ×: 5 pmol, 0.1 ×: 2.5 pmol). Ctrl miRNA was used as a control.

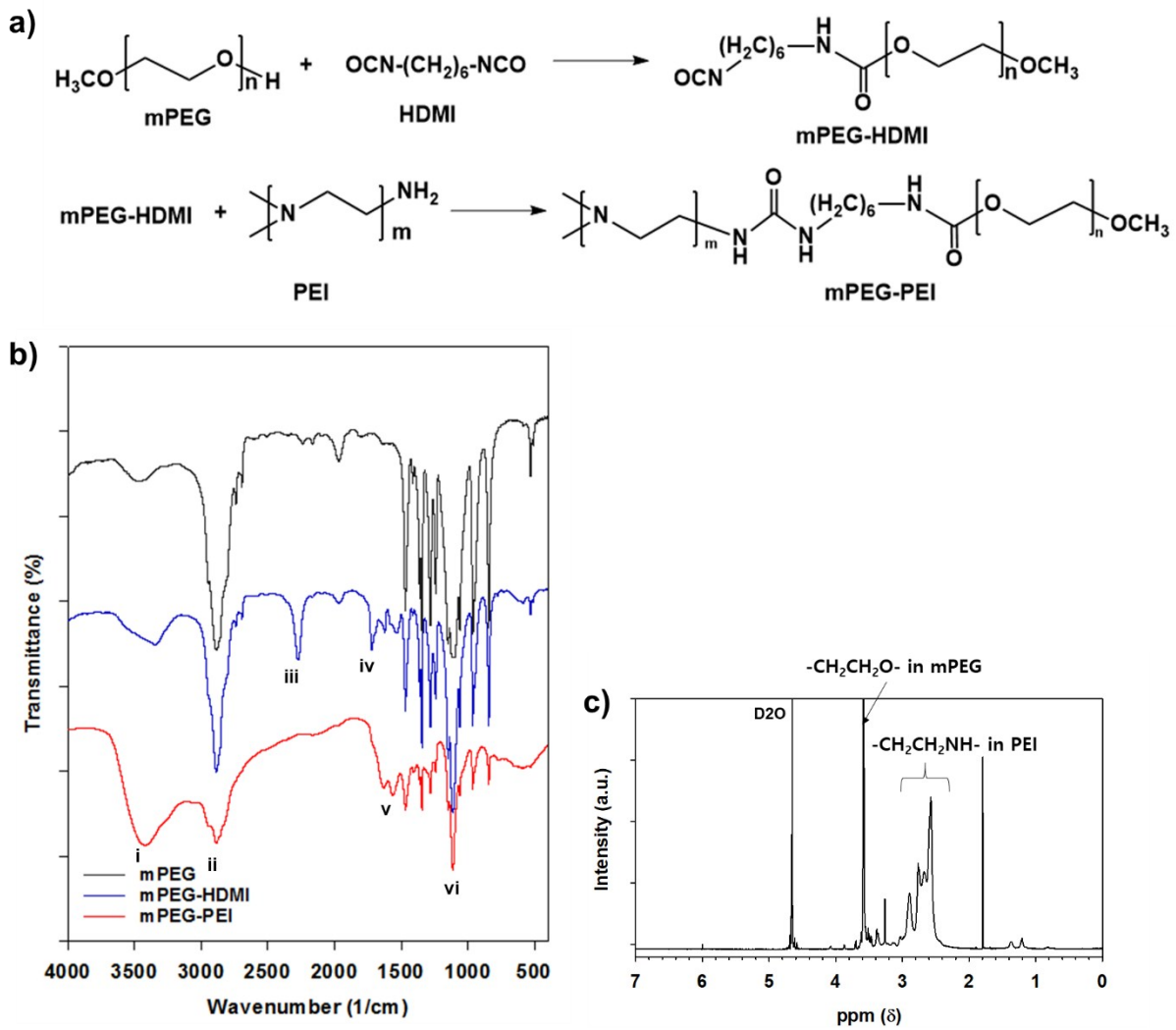

Figure S6. (a) Synthetic scheme of mPEG-PEI. (b) FTIR spectra of mPEG (black), mPEG-HDMI (blue) and mPEG-PEI (red). (c) ^{1}H-NMR spectrum of mPEG-PEI in D_2O. (i: N-H amine stretching, ii: C-H stretching, iii: O=C=N isocyanate stretching, iv: C=O urethane stretching, v: N-H banding, vi: C-O ether stretching).

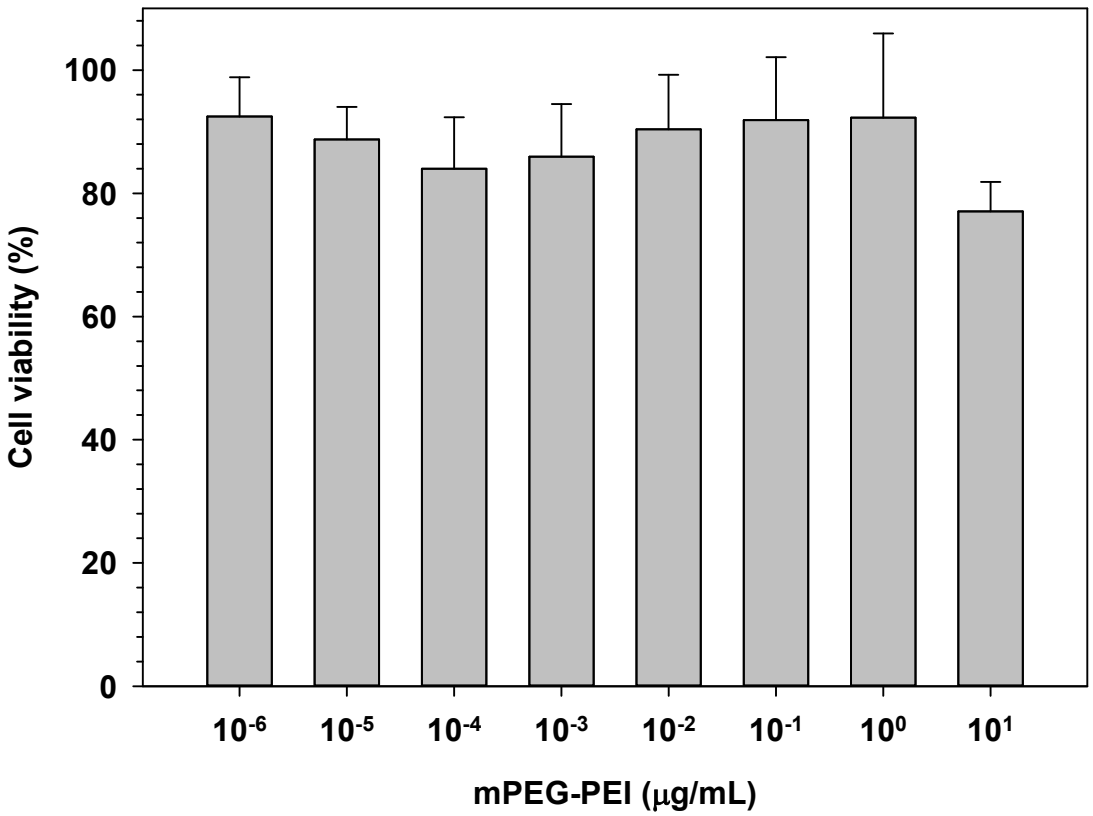

Figure S7. Viability of MDA-MB-231 cells treated with mPEG-PEI at different concentrations ranging from 10^{-6} to 10 μg/mL for 24 hours.

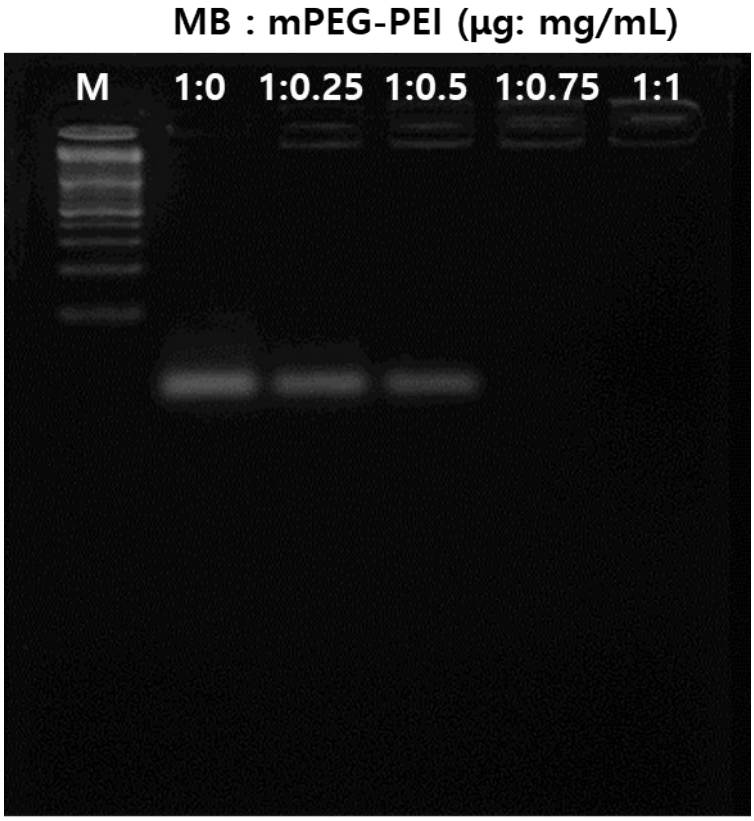

Figure S8. Gel retardation assay of the MBs circuit/mPEG-PEI complexes at various concentration ratios of mPEG-PEI (0, 0.25, 0.5, 0.75, 1 mg/mL) to MB (1 μg). (M: Marker).

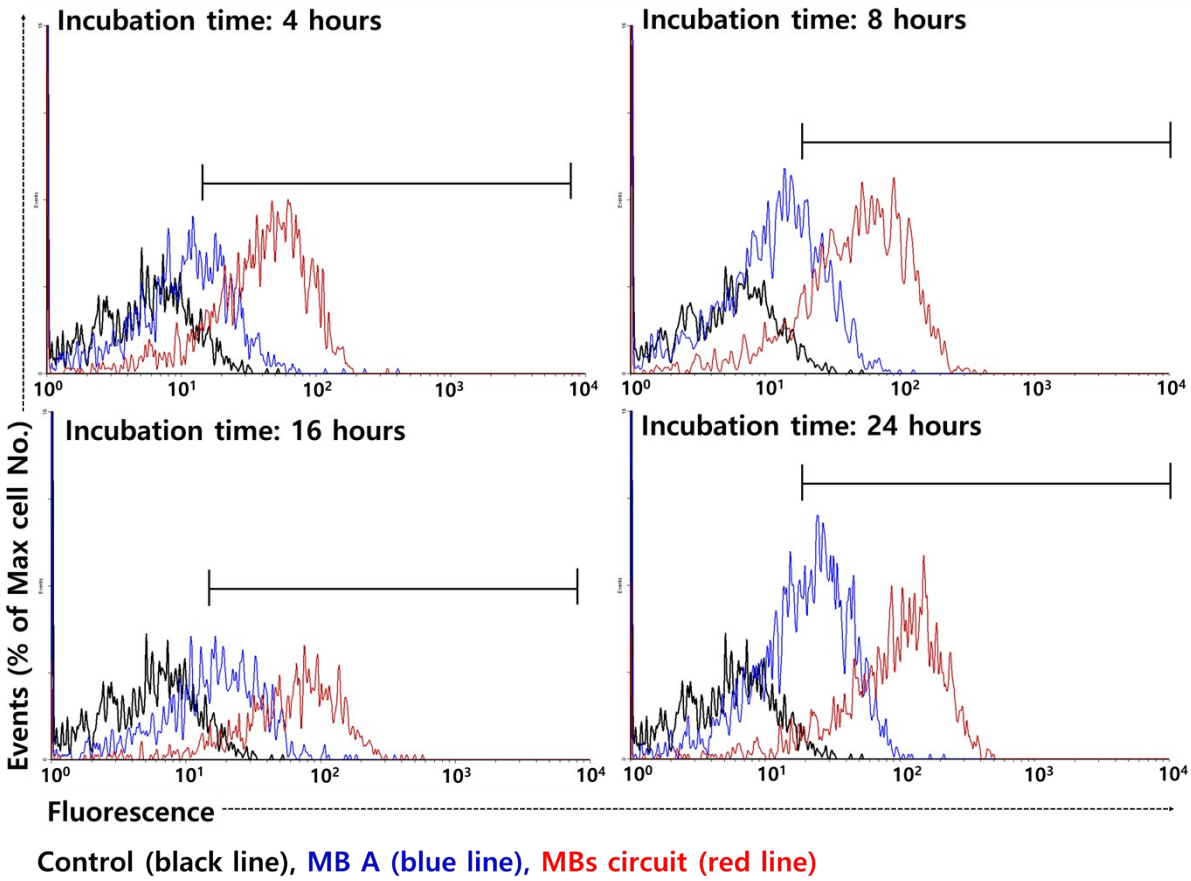

Figure S9. Fluorescence of MDA-MB-231 cells after incubation with MBs circuit and MB A for 4, 8, 16 and 24 hours, respectively, as determined by flow cytometry analysis. (Black: NT, blue: MB A, red: MBs circuit).

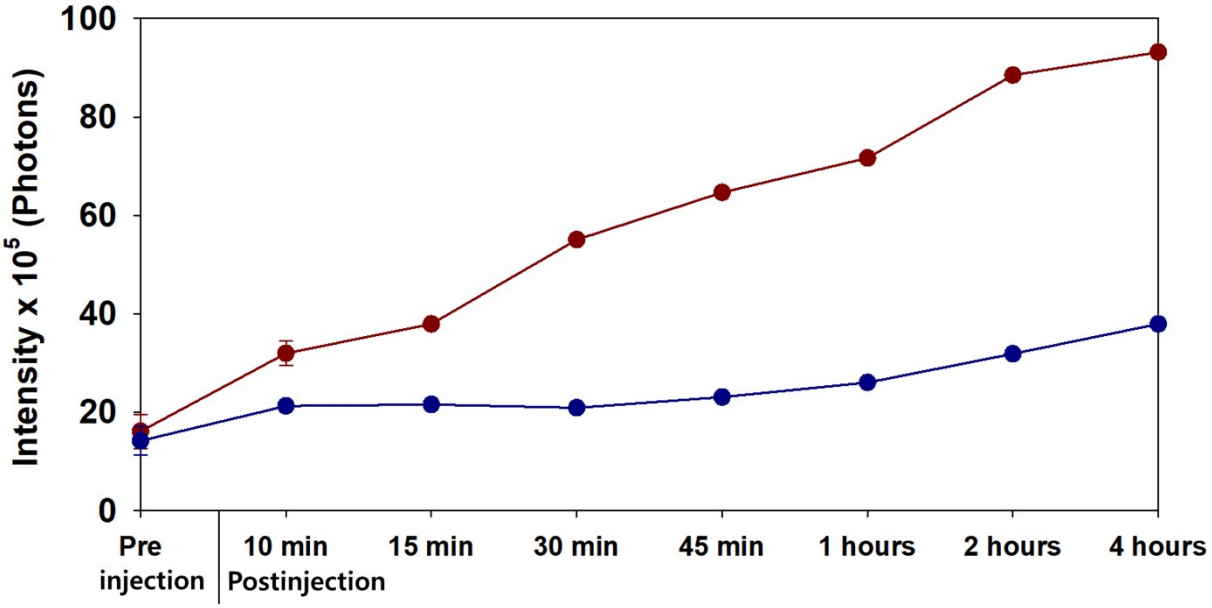

Figure S10. Signal intensity (photon count) in the tumor regions as determined by *in vivo* optical fluorescence imaging over time after the injection of MB/mPEG-PEI complexes (dark red circles) and MB A/mPEG-PEI complexes (dark blue circles).