Supplementary Information

Deoxyribozyme-loaded nano-graphene oxide for simultaneous sensing and silencing of hepatitis C virus gene in liver cells

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MATERIAL AND METHODS

Materials

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Sigma, U.S.A. 10X PBS (Phosphate Buffered Saline), DMEM (Dulbecco's Modified Eagle's Medium), and FBS (Fetal Bovine Serum) were purchased from WELGENE, Korea. SYBR® Gold nucleic acid gel stain was purchased from Life technologies, USA. DNase I was purchased from New England Biolabs, USA. All Dz derivatives and siRNA were purchased from Genotech and Bioneer, Korea.

Preparation of nGO

GO was synthesized from graphite following a modified Hummers method. The dried GO was dispersed in distilled water (3 mg/mL) and the dispersion was placed under bath-sonication for 6 h maintaining the temperature not to excess 40 °C by adding ice to the water bath to obtain nGO. The resulting dark brown solution was filtered through a Büchner funnel and washed with copious amount of distilled water. The yield of the prepared nGO was roughly estimated as ~30-50% based on the weight of obtained nGO per the weight of graphite. The dried filter cake was re-dispersed in distilled water to make the final concentration of 1 mg/ml. The zeta potential of synthesized nGO was measured as -18.2 ± 0.53 mV in PBS (pH 7.4) by a Zetasizer Nano ZS (Malvern instruments, U.K.).

Characterization of nGO

The atomic force microscopy (AFM) analysis was carried out to determine the thickness and lateral size of nGO sheets by XE-100 (Park System, Korea) with backside gold-coated silicon SPM probes (M to N, Korea). UV-vis spectrum of nGO was obtained with a UV-2550 (Shimadzu, Japan). Raman spectrum of nGO was obtained by LabRAM HR UV/vis/NIR (Horiba Jobin Yvon, France) using a 20 mW Ar ion CW laser (514.5 nm) as an excitation source focused through a BXFM confocal microscope equipped with an objective (50X, numerical aperture=0.50). Elemental analysis was carried out by EA1110-FSIONS for carbon and Flash EA 1112 for oxygen.

Loading capacity test

To estimate the loading capacity of Dz to nGO, the fluorescence emission spectra of FAM-Dz and FAM-labelled scrambled-sequence DNAzyme (FAM-scDz) were obtained at excitation wavelength 492 nm with or without nGO in PBS by using a fluorometer (BioTek, U.S.A.). Loading capacity was calculated from the degree of FAM fluorescence quenching by binding of DNAzyme to nGO.

Fluorescence recovery test in buffer solution.

Recovery of fluorescence intensity of Dz/nGO upon addition of target HCV NS3 RNA was measured to elucidate the sequence selectivity of the Dz/nGO for target RNA sensing. FAM-Dz and FAM-scDz (5 pmol) were first mixed with nGO in 50 μl PBS for 10 min, followed by incubation with different molar ratios of target HCV NS3 RNA (1:1, 1:2, 1:4) for 30 min at room temperature in triplicate. The fluorescence emission spectra of FAM were obtained at emission wavelength 520 nm by using a

fluorometer (BioTek, U.S.A.).

To measure the limit of detection (LOD), 50 pmol of FAM-Dz was first mixed with nGO, resulting in 95% fluorescence quenching. A total of 25 µl of FAM-Dz/nGO solution was mixed with 25 µl of target mRNA in a 96-well plate, followed by addition of various concentrations of mRNA (0.02 – 1 µM). The fluorescence emission spectra of FAM were obtained at emission wavelength 520 nm by using a fluorometer (BioTek, U.S.A.). All the samples were prepared in triplicate. We calculated the LOD of FAM-Dz/nGO system by using the equation of "LOD = 3.3(standard deviation/slope of the calibration curve)".

We measured the quantum yields and extinction coefficients of FAM-Dz/nGO with and without target mRNA, where 50 pmol of FAM-Dz and 6 ug of nGO in 300 ul constituted a stock solution and 50 pmol of target mRNA was added if applicable.

DNase protection assay

Polyacrylamide gel electrophoresis (PAGE) retardation assay was carried out to estimate the DNase protection ability of FAM-Dz/nGO complex. Various concentrations of DNase I (1.25 – 10 units) were first incubated with FAM-Dz or FAM-Dz/nGO in 50 µl PBS for 30 min, followed by gel electrophoresis and SYBR gold staining. For the fluorescence detection, the fluorescence emission spectra of FAM-Dz were obtained at emission wavelength 520 nm by using a fluorometer (BioTek, U.S.A.).

Cell culture

Human hepatoma cell line Huh-7 (Naïve Huh7) was grown in DMEM containing 4.5 g/L D-glucose containing 10% FBS, 1% penicillin and streptomycin at 5% CO_2, 37 °C. To maintain Huh-7 cells carrying a subgenomic HCV replicon (genotype 1b) and a luciferase reporter gene (abbreviated as Huh-7-rep), 500 µg/ml of G418 (A.G. Scientific, Inc. USA) was added to the above media.

Cell viability test of nGO

Huh-7-rep cells were seeded in a 96-well plate with a density of 1.0×10^4 cells/well in triplicate for 24 h and then incubated with different concentrations of nGO for 12 h. Next, the nGO treated cells were rinsed with 1X PBS and 200 µl of serum-free media and 20 µl of MTT reagent were added to each well. After 3 h, the MTT solution was removed and each well was rinsed, followed by addition of 200 µl of DMSO to dissolve insoluble purple formazan product. The absorbance was then measured at 560 nm using a microplate reader (Molecular Devices, Inc., USA). For the CCK-8 cell viability assay, we prepared Huh-7-rep cells (1×10^4 cells/well) in a 96-well plate for 24 h, followed by incubation with various concentrations of nGO with serum free medium. After 12 h incubation, the medium was replaced with fresh serum containing medium. The cells were then carefully washed with 1XPBS, then CCK-8 assay solution was added for 1 h, followed by measuring absorbance at 450 and 670 nm using a microplate reader (Molecular Devices, Inc., USA).

Cellular uptake study

Huh-7-rep cells were seeded in a 12-well plate with a density of 1.2×10^5 cells/well. After 24 h

incubation for 70-80 % cell confluency, FAM-Dz and FAM-scDz (500 pmol) loaded nGO complex were added to each well in serum-free media for 12 h at 5% CO_2, 37 °C (final volume was 500 μL). Then, cell media was changed to serum-containing fresh media and incubated for 2 h at 5% CO_2, 37 °C. After washing with 1XPBS, cell nuclei were stained with Hoechst 33342 via manufacturer's protocol at room temperature in dark to protect from light at least 5 min. Then, the cells were rinsed and the media was replaced with fresh media. Cell images were obtained using a Ti inverted fluorescence microscope with a 10X (1.4 numerical aperture) objective (Olympus, Japan).

Semi-quantitative RT-PCR and Western blot

When the cell confluency reached to 70-80% in a 12 well plate (1.2×10^5 cells/well), the cells were treated with Dz and scDz in serum-free media in complex with nGO. For lipofectamine (lipo)-mediated transfection, cells were incubated with Dz or scDz in the presence of lipo for 4 h, the media was removed and the cells were rinsed with 1XPBS. Next, after addition of serum-containing media, the cells were further incubated for 44 h. For the treatment of Dz/nGO complex, 250 pmol and 500 pmol of Dz derivatives were prepared in 1XPBS containing nGO and then added to each well (final volume is 500 μL). And the media was replaced with fresh serum-containing media after 12 h of incubation of cells with Dz/nGO complex, and then the cells were further incubated for 36 h before microscopic and functional evaluation.

For the semi-quantitative RT-PCR (Reverse Transcription Polymerase Chain Reaction), total RNA was collected by using Trizol reagent (Invitrogen) based on manufacturer's protocol. Quantity and quality of the collected total RNA was estimated based on absorbance at 260 nm (A260) and RNA/protein ratio (A260/A280) was measured by using a UV-Vis spectrophotometer (Nanodrop Take3 or fluorometer (BioTek, U.S.A.)). 1000 ng of total RNA was used for reverse transcription by using Superscript II reverse transcriptase (Invitrogen) based on the manufacturer's protocol. The product cDNA was amplified by PCR (BioRad, U.S.A.) using following primer pairs:

(1) HCV NS3 forward primer: 5'-CCT ACT GGT AGC GGC AAG AG-3'
(2) HCV NS3 reverse primer: 5'-CTG AGT CGA AAT CGC CGG TA-3'
(3) GAPDH forward primer: 5'-TTG TTG CCA TCA ATG ACC CCT TCA TTG ACC-3'
(4) GAPDH reverse primer: 5'-CTT CCC GTT CTC AGC CTT GAC GGT G-3'

The PCR reaction for HCV NS3 was performed as follows: 2 min at 95 °C, (60 s at 95 °C, 60 s at 62 °C, 30 s at 72 °C) × 35 cycles. The PCR reaction for GAPDH, used as housekeeping gene, was performed as follows: 5 min at 94 °C, (30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C) × 26 cycles. The product of PCR was separated by 1.2% TAE agarose gel and then analyzed by a Gel Doc (ATTO, Korea). Relative band intensities were quantified by using Image J software and NS3 gene expression level was normalized relative to GAPDH expression level.

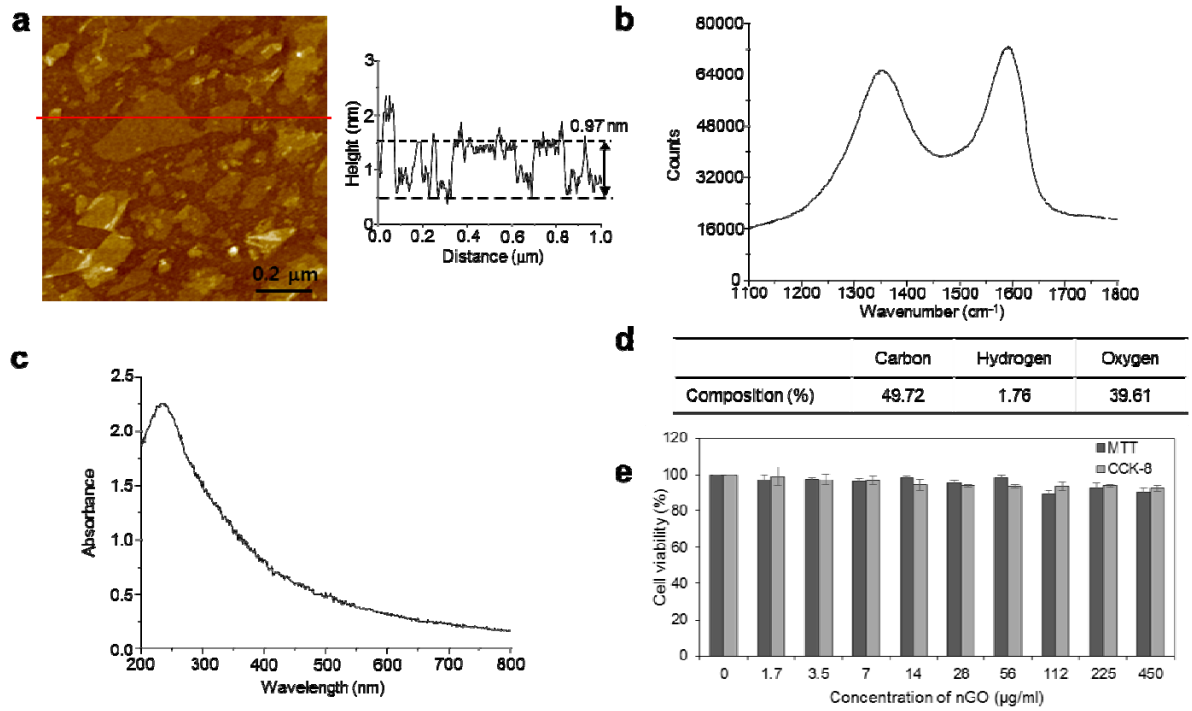
For Western blotting, the cell lysates were separated on SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked with 5% skim milk and probed with primary antibody, anti-HCV NS3 (Virostat, USA) and anti-GAPDH antibody (ABFrontier). The membrane was then incubated with horseradish-peroxidase-conjugated secondary antibody (Sigma) and visualized by a Luminescent Image Analyzer (GE Healthcare, Sweden).

Flow cytometry

Huh-7-rep cells were seeded in a 12-well plate with a density of 1.2×10^5 cells/well. After 24 h incubation for 70-80% cell confluency, FAM-Dz (500 pmol) loaded nGO complex in serum-free media was added to each well and incubated for 12 h at 5% CO_2, 37 °C (final volume was 500 µl). Then, cell media was changed to serum-containing fresh media and incubated for 2 h at 5% CO_2, 37 °C. After washing with 1XPBS, trypsin-EDTA was added to cells for 3 min and then 10% FBS was added to cells, followed by centrifugation (5000 rpm, 3 min). The cells then were washed with 1XPBS. The fluorescence intensity of the cells was measured by a flow cytofluorometer (Beckton Dickinson).

Time-dependent recovery of fluorescence

Huh-7-rep cells were seeded in a 96-well plate with a density of 1.5×10^4 cells/well. After 24 h incubation, a solution of FAM-Dz (50 pmol) loaded nGO complex in serum-free media was added to each well for 12 h at 5% CO_2, 37 °C (final volume was 50 µl). The recovery of fluorescence in the cells was observed by using an in-cell-analyzer 2000 (GE Healthcare Life Sciences) at different time points.



	Carbon	Hydrogen	Oxygen
Composition (%)	49.72	1.76	39.61

Figure S1. Characterization of nGO. a. AFM image and line profile of nGO showed width and height of nGO as approximately 0~300 nm and 0.97 nm, respectively. b. Raman spectrum of nGO showed D and G peaks at 1354 and 1594 cm-1, respectively, giving ID/IG = 0.90. c. UV-vis spectrum of nGO. d. Elemental analysis to estimate the content of each elements of nGO. e. Cell viability of Huh-7-rep cells incubated with different concentrations of nGO as mean values from triplicates.

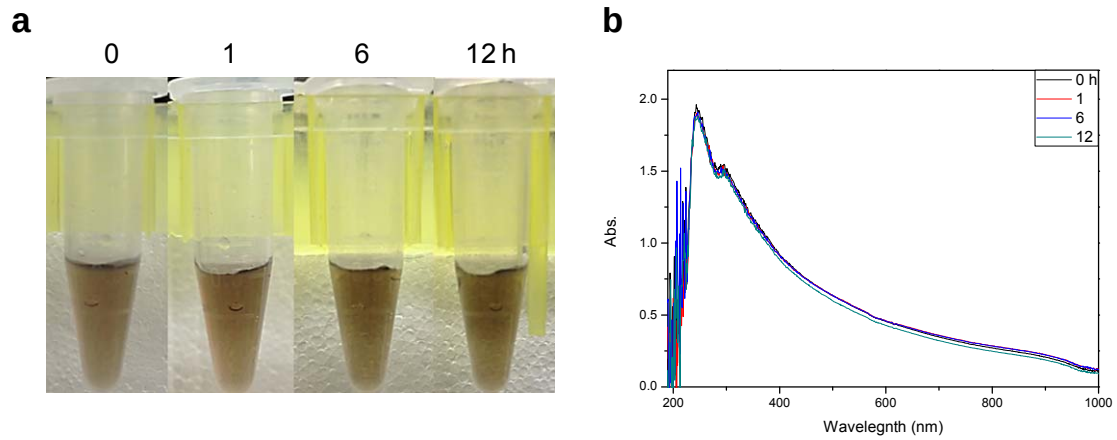


Figure S2. NGO stability test. **a.** Photograph of Dz/nGO complex in PBS containing serum. NGO maintained colloidal stability after Dz loading in the serum containing PBS solution up to 12 hrs. **b.** UV-Vis absorption spectra of Dz/nGO solution showed no changes in intensities of the pi-pi* transition peak at 230 nm for 12 h. The absorption peak at 300 nm and saturation below 230 nm were derived from components of serum. This result showed that the prepared nGO is stable after Dz loading in the presence of serum under physiological conditions at least for 12 hrs.

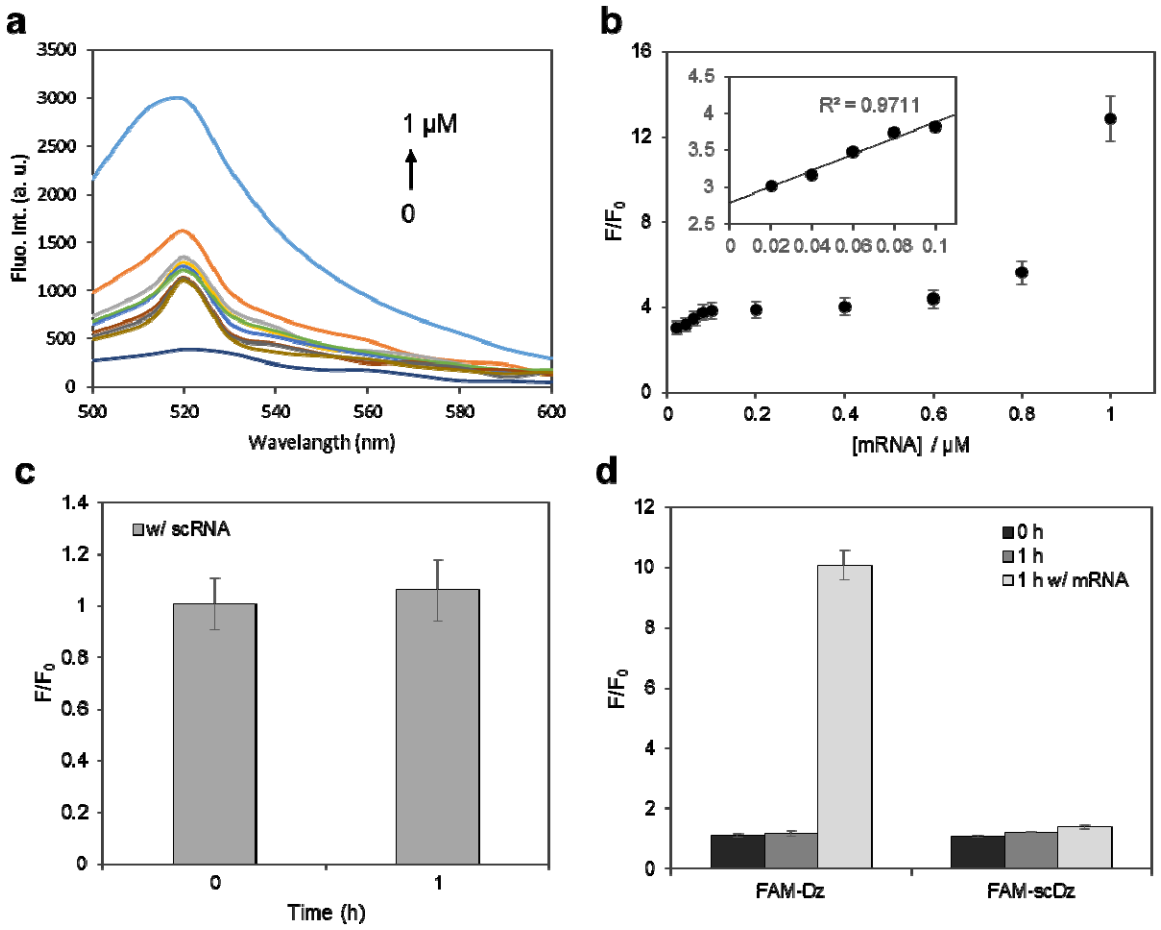


Figure S3. The fluorescence intensity of FAM-Dz. **a,b**.The quenched fluorescence of FAM-Dz by nGO was recovered increasingly upon the addition of the complementary mRNA in a concentration-dependent manner (0.02 – 1 μM). **c.** The quenched fluorescence of FAM-Dz by nGO was stably maintained even after incubation with scRNA in the presence of serum for 1 hr. **d.** The quenched fluorescence of FAM-Dz by nGO in the presence of serum was significantly recovered with addition of target mRNA but FAM-scDz/nGO did not show significant fluorescence increase with addition of mRNA.

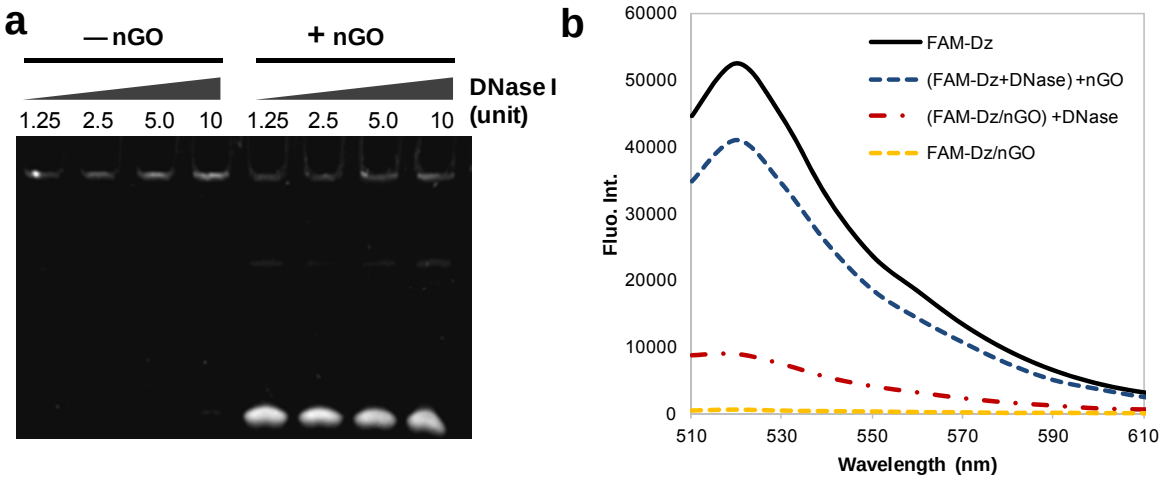


Figure S4. DNase protection assay. a. PAGE gel retardation assay for Dz incubated with different amounts of DNase (1.25, 2.5, 5.0, 10 units) in the presence or in the absence of nGO. Without nGO, Dz was completely degraded by DNase I whereas the Dz adsorbed to nGO was resistant to the DNase I-mediated cleavage. b. Fluorescence spectra of FAM-Dz after treated with DNase I, with or without nGO. Degradation of Dz by DNase I was inhibited by nGO, showing much less decrease of the fluorescence intensity upon treatment of DNase I in the presence of nGO compared to that of the Dz treated with DNase I in the absence of nGO, followed by addition of nGO.

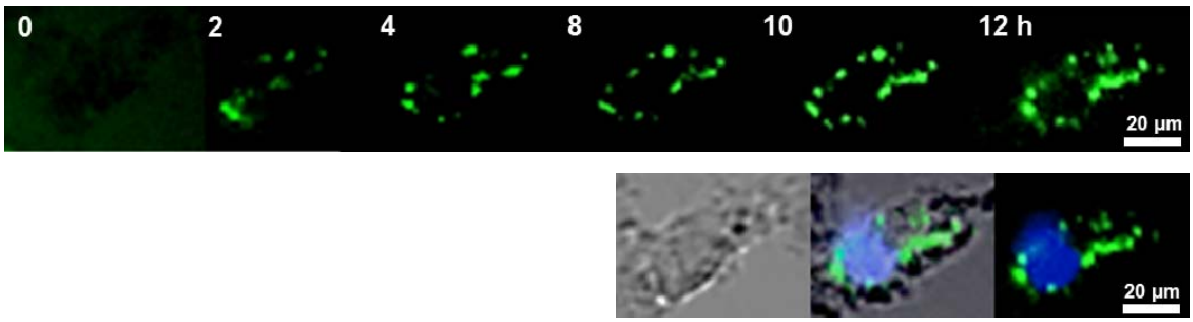


Figure S5. Fluorescence recovery in Huh-7-rep cell was observed over time after treatment with 1 μM of FAM-Dz/nGO. The fluorescence of FAM-Dz gradually increased as time went by (0 – 12 h) in cytoplasm. Blue: nucleus stained with Hoechst 33342, Green: FAM.

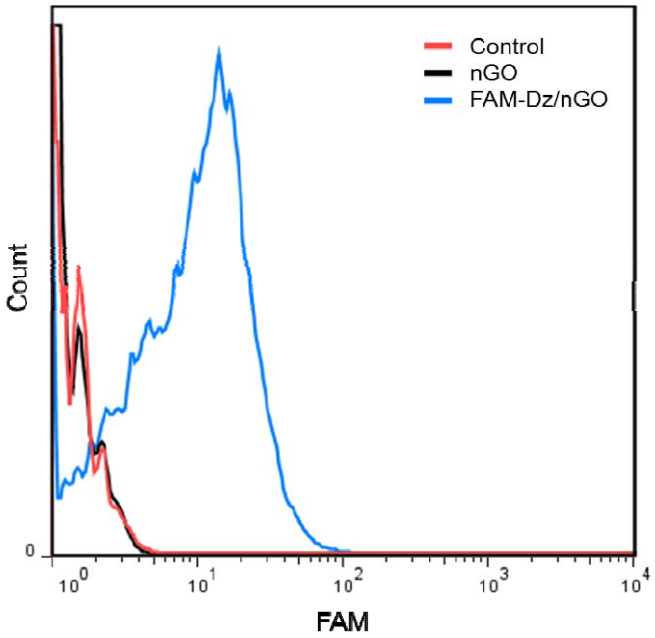


Figure S6. For quantitative measurement of fluorescence recovery in live cells, flow cytometry was carried out. The mean fluorescence of FAM significantly increased in the cells treated with FAM-Dz/nGO compared to the control cells treated with only nGO or the cells without any treatment.

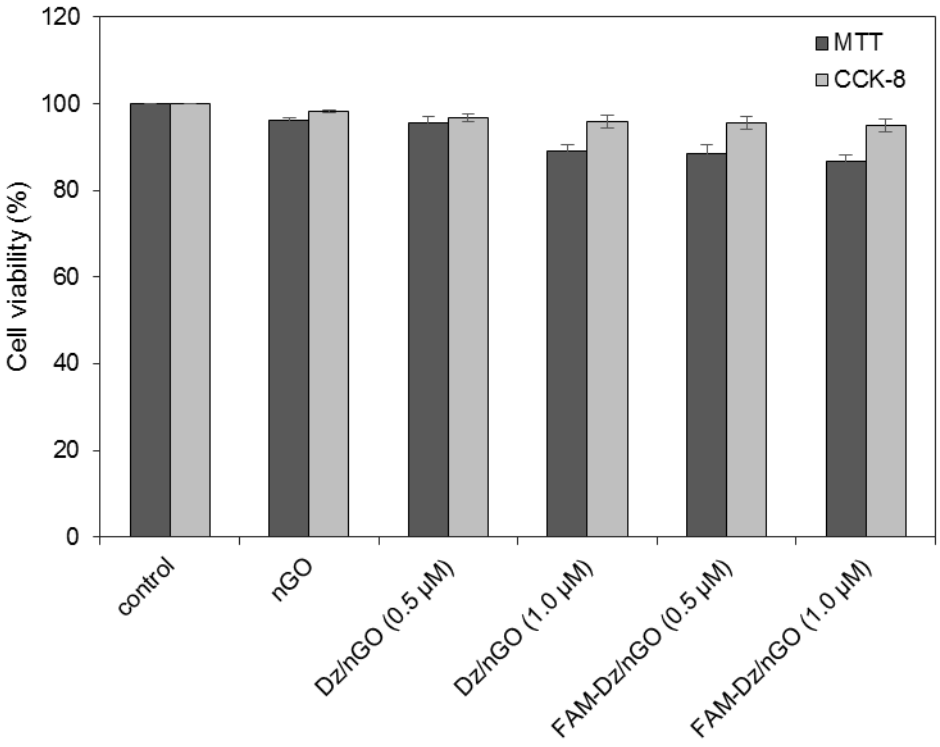
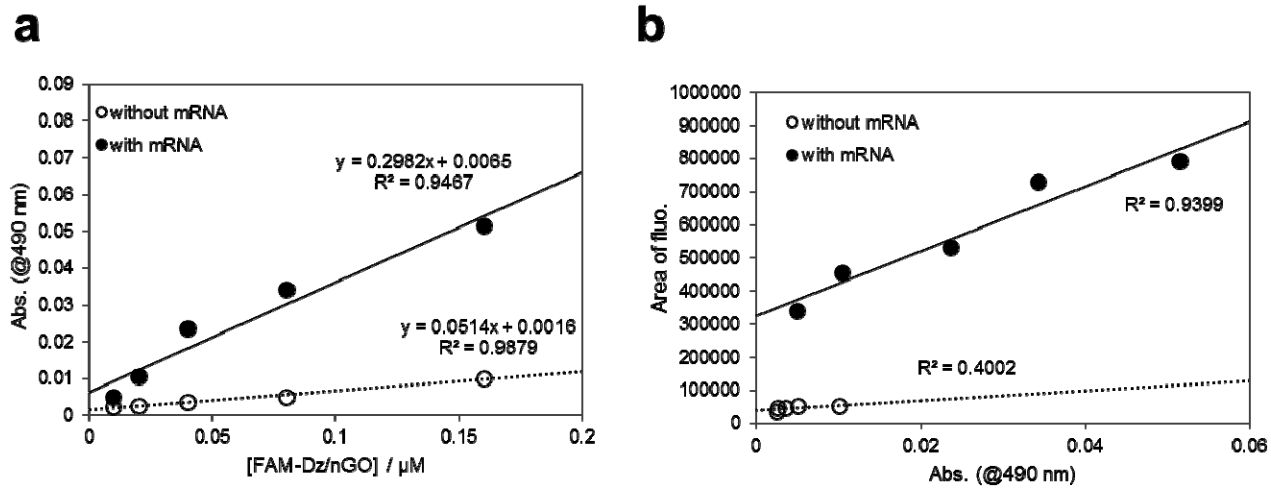


Figure S7. The viability of Huh-7-rep cells after treatment with Dz/nGO or FAM-Dz/nGO complex for 12 h in a 96- well plate. Both assay results showed that ~100% of cell viability was maintained even after treatment of Dz/nGO or FAM-Dz/nGO complex with concentrations used in the present study.



	$\varepsilon_{490\,nm}$, extinction coefficient (L/mol*cm)	QY, quantum yield, 520 nm
FAM-Dz/nGO with mRNA	298,200	0.88
FAM-Dz/nGO without mRNA	51,400	0.14

Figure S8. The extinction coefficient and quantum yield of FAM-Dz/nGO were measured in the presence and absence of target mRNA. A total of 150 μl of FAM-Dz/nGO solution (0.01 – 0.16 μM) was mixed with 50 pmol of target mRNA (final volume = 300 μl) for 1 h, followed by measuring absorbance spectra. And the fluorescence spectra were measured at excitation wavelength 490 nm. **a.** Absorption spectra of FAM-Dz with and without mRNA, **b.** Area of fluorescence vs. Absorbance.