# Electronic supplementary information (ESI)

## Fluorescent imaging of cytoplasmic nucleolin in live cells by functionalized-engineered aptamer

Junfa Yin,[a,b] Shaokun Chen, [a,b] Yuanyuan Song [a] and Hailin Wang [a,b]*

[a] *State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, PR China. E-mail: hlwang@rcees.ac.cn, Tel: +86-10-62849611*

[b] *University of Chinese Academy of Sciences, Beijing, 100049, PR China.*

## 1. Preparation and characterization of graphene oxide (GO) and polymer-grafted GOs

Graphene oxides were synthesized by a modified Hummers method (1) from natural graphite powder. Typically, graphite powder (2 g) was added to $H_2SO_4$ (98%, 50 mL) and then stirred for 2 h. Afterwards, 10 g of $KMnO_4$ was added under stirring and the temperature of the mixture was kept to less than 30 °C for 4 h. Distilled water (100 mL) was added and the mixture was stirred at room temperature for another 4 hours. The reaction was terminated by the addition of distilled water (400 mL) and $H_2O_2$ (30%, 25 mL) solution. The mixture was repeated washed with 1M HCl aqueous solution (100 mL x 3), and then distilled water (200 mL x 3). Exfoliation was conducted by sonicating graphene oxide (typically 2 mg $mL^{-1}$) dispersion under a power of 40W for 4 h. The resulted sample was centrifuged at 6000 rpm for 5 min, and the upper solution was taken for further experiments.

Polymer grafted GO nanocomposites, including PEI-*g*-GO, PEG-*g*-GO and PEG($NH_2$)-*g*-GO, were prepared as previously described (2,3), by taking advantages of the abundant carboxyl groups (-COOH) in GO nanosheets. Briefly, 0.5 mg/mL GO solution (20 mL) were activated with 1.5 mM N-hydroxysuccinimide (NHS) and 1.5 mM 1-ethyl-3-(3-(dimethylamino) propyl)carbodiimide hydrochloride (EDC), followed by sonication for 30 min. Afterward, polymer (22 mg of polyethyleneimine, Mn=5000 Da, for PEI-*g*-GO; 30 mg of polyetheylene glycol-amine, Mn=5000 Da, for PEG-*g*-GO; and 20 mg of 4arm-PEG-Amine, Mn=2000 Da, for PEG($NH_2$)-*g*-GO) were added under stirring (2000 rpm) for 12 h. The reaction was terminated by adding 5 mL of 2-mercaptoethanol solution (10%). Finally, all GO materials were dispersed in sterilized deionized water. The addition of 0.2% ammonia can effectively prevent the aggregation and precipitation of polymer-*g*-GOs in a high concentration of 2 mg/mL, thus increasing the stability of these polymer-*g*-GOs solution within two weeks. Prior to use, the ammonia can be easily neutralized by addition of acetic acid, and polymer-*g*-GOs was diluted into a typical concentration of 0.05 mg/mL.

The morphology of GO and polymer-*g*-GO nanosheets was characterized by TEM with H-7500 transmission electron microscope (Hitachi Scientific Instruments, Japan). With respect to XPS determination, GO samples were deposited onto silicon wafers, air-dried, and then the silicon wafers were subjected to ESCALAB 250Xi X-ray photoelectron spectroscopy (Thermo Fisher, USA). TGA analysis was performed on an SDT Q600 thermobalance

(TA Instruments) under nitrogen gas flow (100 mL/min) at a heating rate of 10 °C/min using alumina crucibles.

**2. Cell Culture**

Human lung carcinoma epithelial A549 and fibroblast normal MRC-5 cells (supplied by cell culture centre, institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM)–high glucose (Hyclone, CE Healthcare Life Sciences) supplemented by 10% fetal bovine serum (FBS, Gibco® Life technologies), 1% penicillin and streptomycin (Corning). Cells were grown in 5% $CO_2$ in air-humidified incubator at 37°C, in which media were replaced every 48 h. The cells were subjected for transfection and laser scanning confocal microscopy (LSCM) imaging when they were approximately 60-80% confluent.

**3. Confocal fluorescent imaging of nucleolin in fixed and live cells**

*Fixed cells*: A549 and MRC-5 cells were grown on 8-chamber glass-bottom dishes (Thermo Fisher, USA) for 24 h, and then fixed in ice-cold 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Afterwards, the cells were stained by 100 nM of Fam-AS1411/PEG($NH_2$)-*g*-GO nanocomposites in DMEM medium for 40 min. The cells were counterstained with DAPI to label nuclei, DiD to label plasma membrane for 10 min, and then visualized by confocal fluorescence microscopy.

*Live cells*: A549 and MRC-5 cells were seeded on 8-chamber glass-bottom dishes and incubated for one day. The cells were stained by 100 nM of Fam-AS1411/PEG($NH_2$)-*g*-GO nanocomposites for 40 min. The intracellular nucleolin can thus be directly visualized by confocal fluorescence microscopy.

**4. Immunofluorescent imaging of nucleolin in A549 and MRC-5 cells**

For confocal fluorescence and colocalization experiments, A549 and MRC-5 cells were first stained by 100 nM of Fam-AS1411/PEG($NH_2$)-*g*-GO nanocomposites for 40 min. The stained cells were fixed in 4% paraformaldehyde for 10 min on ice, washed with BSS buffer (135 mM NaCl, 2 mM KCl, 1.1 mM $Na_2HPO_4$,1.9 mM $KH_2PO_4$, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.2), and permeabilized with 0.1% Triton X-100 for 5 min. Afterwards, the cells were blocked with 5% non-fat dry milk in TBST (10 mL Tris-HCl pH 7.5, 8.8 g NaCl,1 mL Tween-20 in 1 L water) for 40 min, incubated for 3 h with primary antibodies anti-nucleolin (ab22758, Rabbit polyclonal to nucleolin). Washed with BSS Buffer 3 times, the cells were then

incubated for 1 h with fluorescent secondary antibodies Fluorescein-conjugated affiniPure Donkey Anti-Rabbit IgG (H+L) (ab10051, in green fluorescence), and washed again as before. Slides were mounted with Vectashield containing DAPI and imaged on a Leica SP5 confocal microscope at 40X or 63 X magnifications under oil immersion.

**5. Cytotoxicity Assay**

For cytotoxicity assay, 2.0 × $10^4$ A549 cells/well were seeded in 96-well plates, respectively, and incubated in a humidified 5% $CO_2$-balanced air incubator at 37 °C. Thereafter, cells were treated with different Fam-AS1411 aptamer (polymer-*g*-GO nanosheets, 0.05 mg/mL) at 10, 50, 100 and 200 nM, followed by toxicity assessments. Cell viability was determined through CCK-8 assay with 96-well plates following the instructions from the manufacturers (Shanghai Sangon Biological Engineering Technology and Service Co., China). Cell density and morphology were visualized with Axiovert 40 CFL microscope (Carl Zeiss, Germany).

The influences of GO and polymer grafted GO nanosheets on integrity of cell membrane were determined by LDH assay. Briefly, 1 × 104 A549 cells/well were seeded in 96-well plate, and then incubated with nanosheets at 0.01, 0.05 and 0.10 mg/mL for 24 h. Afterwards, the culture medium was collected from each well, and centrifuged and collected for LDH assessment using LDH Assay Kit (ab65391, Abcam). Absorbance was measured at 490 nm on a Varioskan Flash Multimode plate reader. A549 cells treated with 1% Triton X-100 were used as the positive control (Pos ctrl), and untreated A549 cells were used as the negative control (Ctrl).

**6. Cytoplasmic proteins extraction and Western blot**

Add protease inhibitors (1:100 dilution) and DL-Dithiothreitol (DTT) (1:1000 dilution) to CER I (Thermo Scientific) before use to maintain extract integrity and prevent oxidation. The adherent cells were harvested with trypsin-EDTA (Hyclone CE Healthcare Life Sciences) and centrifuged at 1000 × g for 5 min. After washing with BSS buffer, the cells was transferred to 1.5 mL microcentrifuge tube and centrifuged at 1000 × g for 3 min. The supernatant was carefully removed and discarded, leaving the cell pellet as dry as possible. After adding ice-cold CER I (100 µL) to the cell pellet, the tube was suspended for 15 seconds and incubated on ice for 10 minutes. Then the tube was added ice-cold CER II (5.5 µL) and incubated for 1 minute. At last the tube was centrifuged for 5 min at maximum speed in a

microcentrifuge (16,000 ×g) and the supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube. Protein concentrations were measured using the Bradford assay.

The amount of 30 μg of cytoplasmic proteins was subjected to SDS-PAGE on a 10% polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The blotted PVDF membrane was blocked in freshly prepared 5%-nonfat dry milk for 1 h at room temperature with constant agitation, then incubated with anti-nucleolin (ab22758, Rabbit polyclonal to nucleolin) and β-tubulin antibodies (sc-9935, 1: 200 dilution) for 2 h at room temperature. Western blotting signals were detected by BIO-RAD ChemiDoc XRS chemiluminescence system (Bio-Rad, USA).

**7. siRNA transfection**

The siRNA transfection experiment was performed in A549 cells with Lipofectamine 2000 (Thermo Fisher Scientific). A549 cells were plated 12 h before siRNA transfection at a density of ~2 x $10_5$ cells per 8-well culture area and transfected with 100 pmol siRNA (UAAUUCAGGAGCAGAUUUGUU). A non-targeting scrambled siRNA (GCUAGCUUUAUUCGUAUAUUA, synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., China.) was used as a negative control (NC). Cells were incubated in this final mixture for 12 - 24 h at 37 $^0$C. Afterward, DMEM with 10% fetal bovine serum and antibiotics was added to the cells and incubated for 72 h at 37 $^0$C. Then the cells were treated with 100 nm of Fam-AS1411/PEG($NH_2$)-*g*-GO for 40 min.Then the medium was removed, the cells were counterstained with DAPI after they were fixed in ice-cold 4% paraformaldehyde and visualized by confocal fluorescence microscopy. The efficiencies of siRNA-depression nucleolin expression were evaluated by fluorescence spectrometry using a spectral scanning multimode reader.

## References


(1) N.I. Kovtyukhova, P.J. Ollivier, B.R. Martin, T.E. Mallouk, S. A. Chizhik, E.V. Buzaneva, A.D. Gorchinskiy, *Chem. Mater.*, 1999, **11**, 771.

(2) Z. Liu, J.T. Robinson, X. Sun, H. Dai, *J. Am. Chem. Soc.*, 2008, **130**, 10876.

(3) M. Xu, J. Zhu, F. Wang, Y. Xiong, Y. Wu, Q. Wang, J. Weng, Z. Zhang, W. Chen, S. Liu, *ACS Nano*, 2016, **10**, 3267.

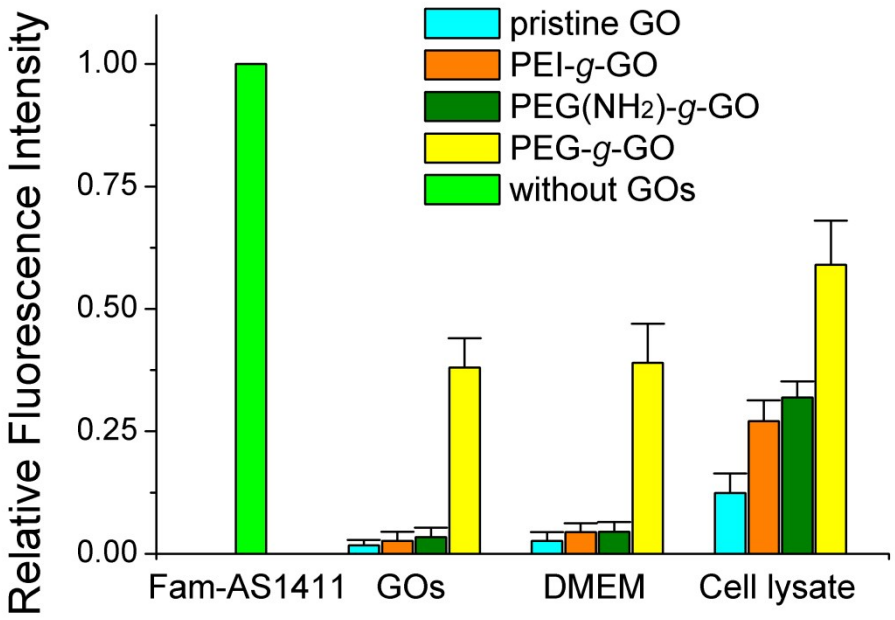


**Figure S1.** Binding and release of Fam-AS1411 probe (200 nM) with/from GO and polymer-grafted-GO nanosheets (0.05 mg/mL). In a concentration range (10 -200 nM) of Fam-AS1411, more than 95% of aptamer binds to GO and polymer-*g*-GO nanosheets, except PEG-*g*-GO nanosheets (< 65%). DMEM media containing 10% FBS for cells culture does not obviously influence the stability of Fam-AS1411/GOs complexes. The bound Fam-AS1411 (on polymer-*g*-GO nanosheets) can be partially released by incubation with nucleolin-containing lysates from A549 cells.

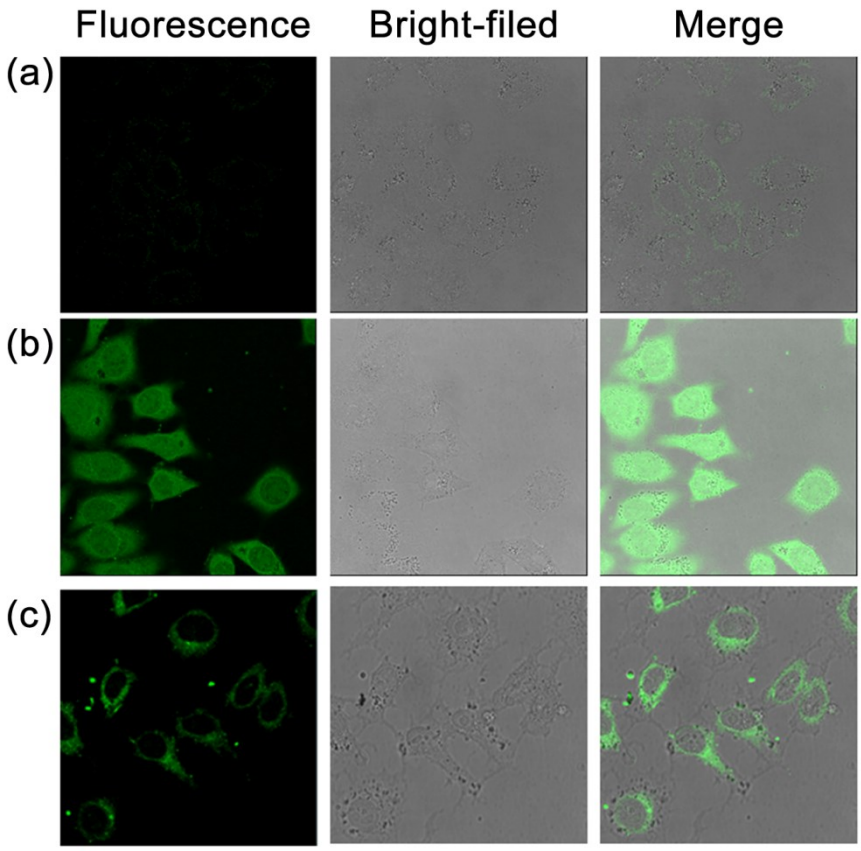


**Figure S2.** Confocal fluorescent images of A549 live cells that were stained with 100 nM of Fam-AS1411 aptamer transtected by 0.05 mg/mL of (a) pristine GO, (b) PEI-*g*-GO, and (c) PEG($NH_2$)-*g*-GO nanosheets.

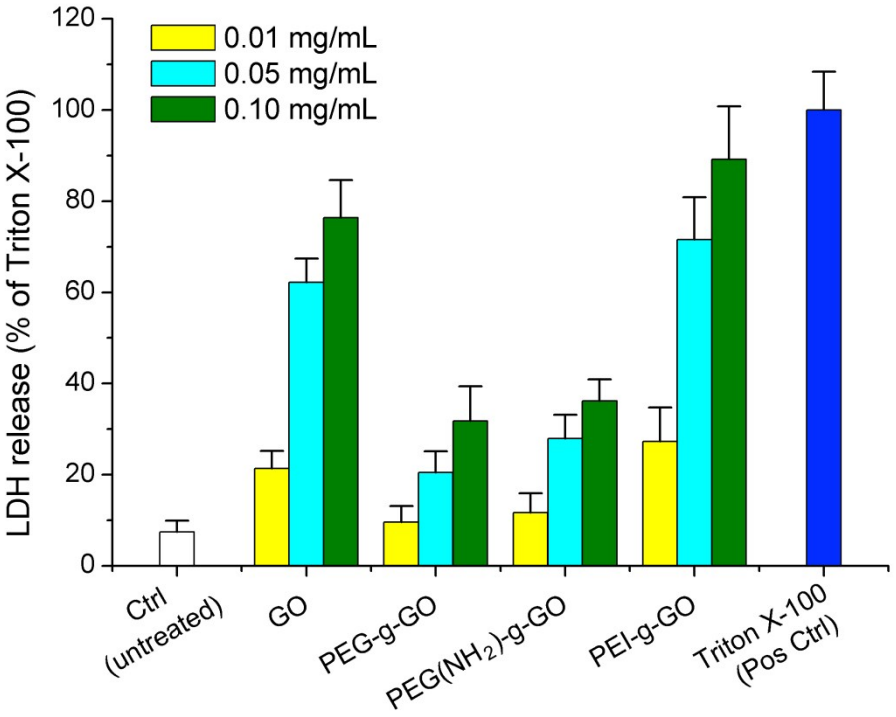


**Figure S3.** LDH release from A549 cells treated with GO and polymer grafted GOs nanosheets at 0.01, 0.05 and 0.10 mg/mL for 24 h (n = 5). A549 cells treated with 1% Triton X-100 were used as the positive control (Pos ctrl), and untreated A549 cells were used as the negative control (Ctrl).

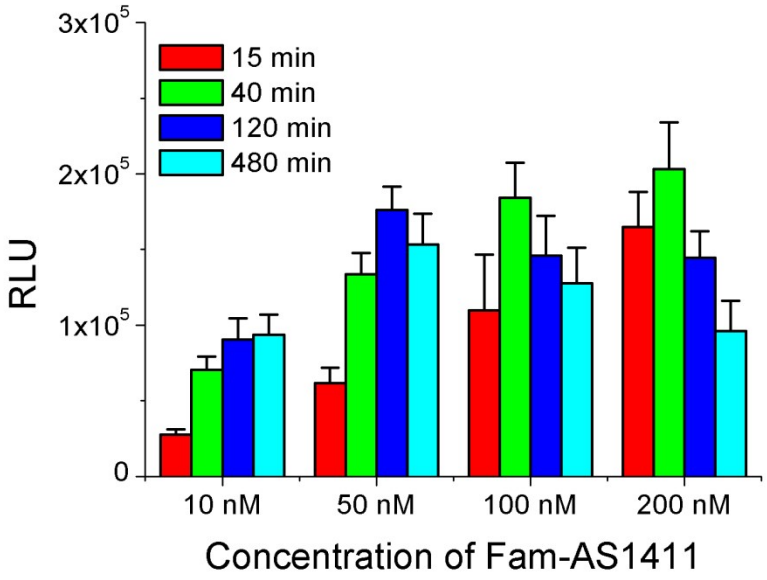


**Figure S4.** Dosage effects of Fam-AS1411 probe (10 - 200 nM) on fluorescence responses (relative light units, RLU) of Fam-AS1411-nucleolin complex in A549 cells. The concentration of PEG($NH_2$) -*g*-GO nanosheets (50 - 220 nm) is 0.05 mg/mL.

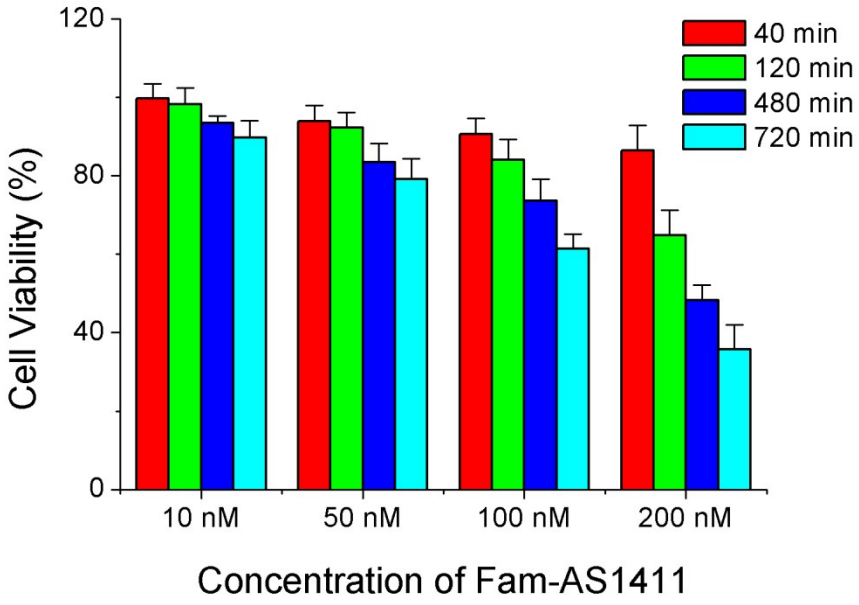


**Figure S5.** Cell viability of A549 cells exposed to Fam-AS1411/ PEG($NH_2$) -*g*-GO nanocomposites at 10, 50, 100, and 200 nM for 40, 120, 480 and 720 min (n = 3). Cell viability was evaluated with CCK-8 assay method.

**Table S1.** Fractioning of polymer-grafted-GO naosheets into three fractions in terms of their 2-D planar sizes by simple filtration.

| Size (nm) [a] | Preparation methods | Proportion (%, in mass) |
|---|---|---|
| 220 ~ 1200 (Fraction 1) | Fraction between PVDF (Millipore™ , 0.22 μm pore size) and PC (Whatman™, 1.2 μm) membrane filters | 56.7±19.6 |
| 50 ~ 220 (Fraction 2) | Fraction between Anodisc AAO (Whatman™, 50 nm pore size) and PVDF membrane filters (Millipore™ , 0.22 μm pore size) | 30.2±9.1 |
| < 50 (Fraction 3) | Filtered by Anodisc AAO membrane (Whatman®, 50 nm pore size) | 12.4±5.2 |