Electronic Supplementary Information

Common Anchor Facilitated GO-DNA Nano-System for Multiplex MicroRNA Analysis in Live Cells

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Calculation of chemical grafting efficiency, η

1. **<u>η of a double-labelled single strand DNA probe.</u>**

The chemical grafting efficiency was calculated by a comparison method. Three parallel experiments were carried out under same conditions:

(1) Sample group: the amine functionalized Cy3-probe21-amine (5'-Cy3-T CAA CAT CAG TCT GAT AAG CTA AGA GAA AGT CAA AGA TGA-NH_2) (1 μM) was reacted with GO (50 μg/mL) in MES buffer (pH 6.5, 25mM) through NHS/EDC coupling.

(2) Control group-1: the Cy3-probe21(1 μM) without the amine functional group was mixed with GO (50 μg/mL) in MES buffer (pH 6.5, 25mM), and the same reagents as used in the sample group. The control group-1 will mimic the physical adsorption of GO to the DNA probe.

(3) Control group-2: the Cy3-probe21(1 μM) without the amine functional group was mixed with the same reagents as used in the sample group in MES buffer (pH 6.5, 25mM), just getting rid of GO.

All the three parallel experiments were carried out at room temperature under constant shaking for 3 h. Thereafter, the same purification methods were applied to. The samples were centrifuged and the supernatants were collected. The residues were washed twice by HEPES/ NaCl/ $MgCl_2$buffer, incubated with HSD/ HEPES/ NaCl/ $MgCl_2$ buffer for 30 min,and washed twice by HSD/ HEPES/ NaCl/ $MgCl_2$ buffer. For each group, the supernatants of all the steps were collected and combined together. The fluorescence of the

combined supernatants was measured respectively. (a) I_S:the fluorescence intensity of the "sample group"; (b) I_{c1}, the fluorescence intensity of the "control group-1";(c) I_{c2}, the fluorescence intensity of the "control group-2".

η was calculated according to the following equation:

$$\eta = \frac{I_{c1} - I_S}{I_{c2}} \times 100\%$$

2. η of a hybrid between the anchor sequence and the DNA probe.

The chemical grafting efficiency was calculated by a comparison method. Three parallel experiments were carried out under same conditions:

(1) Sample group: The mixture of the anchor sequence (1 μM) and Cy3-probe21(1 μM) was annealed at 95 ºC for 10 min and slowly cooled to room temperature. The resultant hybrid is a partial duplex structure with an amine functional group. The annealing hybrid was used to react with GO (50 μg/mL) in MES buffer (pH 6.5, 25mM) through NHS/EDC coupling.

(2) Control group-1: The mixture of the anchor-control sequence (5'-TCA TCT TTG ACT TTC TCT, without amine functionalization compared with the anchor sequence) (1 μM) and Cy3-probe21 (1 μM) was annealed at 95 ºC for 10 min and slowly cooled to room temperature. The resultant hybrid is a partial duplex structure without the functional group. The annealing hybrid was used to mixed with GO (50 μg/mL) in MES buffer (pH 6.5, 25mM), and the same reagents as used in the sample group. The control group-1 will mimic the physical adsorption of GO to the hybrid.

(3) Control group-2: The mixture of the anchor-control sequence (5'-TCA TCT TTG ACT TTC TCT, without amine functionalization compared with the anchor sequence) (1 μM) and Cy3-probe21(1 μM) was annealed at 95 ºC for 10 min and slowly cooled to room temperature. The resultant hybrid isa partial duplex structure without the functional group. The annealing hybrid was mixed with the same reagents as used in the sample group in MES buffer (pH 6.5, 25mM), just getting rid of GO.

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η was calculated according to the following equation:

$$\eta = \frac{I_{c1} - I_S}{I_{c2}} \times 100\%$$

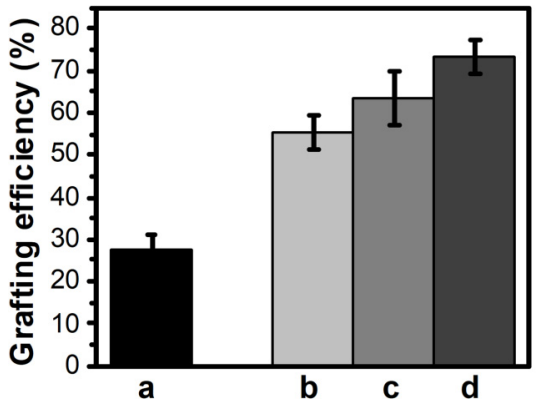

Fig. S1 Chemical grafting efficiency of different kinds of DNA probes to GO. (a) Cy3-probe21-amine (a single-strand DNA probe); (b) ~ (d) the hybrids between the common anchor and each dye-labelled probe, i.e. Cy3-probe21, FAM-probe125b, and Cy5-probe7a, respectively.

Calculation of the limit of detection

The limit of detection (LOD) was carefully calculated according to the 3 σ rule which has been widely used in analytical chemistry. The calculation of LOD for the detection of miRNA-21 is taken as an example here. As the concentration of miRNA-21 was increased from 0 to 1000 nM, the fluorescence of the GBNanos increased correspondingly. A calibration curve was fitted from the linear part (0-50 nM) of titration curve with an equation of $F_{565\ nm} = 100.92678\ [miRNA\text{-}21] + 6096.0371$. The standard deviation σ is 6.089, slope of the standard curve is 100.92678. According to the 3 σ rule, the LOD is calculated to be 181 pM.

LOD = 3σ/slope of the curve = 3 × 6.089/100.92678 = 0.181 nM = 181 pM.

LOD of the other two miRNAs were calculated by the same method. They are 136 pM for miRNA-125b and 210 pM for miRNA-let 7a.

The sensitivity is suitable for monitoring the cellular levels of the majority of miRNA species.

Biosensors which were developed in some recent studies, showing comparable LODs to that of our GO-DNA nano-system, have been successfully applied for intracellular detection of miRNAs. In addition, the comparison between GBNano-*mono*-sc (GBNano was mono-modified with a strand of scrambled DNA with no probing capability) and GBNano-*mono*-21 can provide a straightforward evidence. As shown in Figure 3 of the Maintext, MCF-7 cells treated with GBNano-*mono*-21 showed bright fluorescence in the cytoplasm; while in control experiments, there was no noticeable fluorescence observed from MCF-7 cells incubated with GBNano-mono-sc. These indicate that a LOD of ~ 200 pM of a biosensor is enough for realizing intracellular detection of miRNAs, which may be based on the following reasons.

i. miRNAs are unevenly distributed in cells; therefore, the intracellular detection will be different from the test in tube which is based on a homogeneous solution. Generally, the intracellular miRNA copies range from dozens to thousands of

copies; the regions localized with more miRNAs will offer more chance for signal turning on.

ii. The incubation time for *in vitro* and intracellular detections are different (with detailed description in the "Experimental Part" of Maintext), which may result in different detection limits. For *in vitro* experiment, the incubation time between GBNano and targets was optimized to be 30 min according to the signal-to-noise ratios. For the intracellular experiment, as the cellular uptake will take some time and the diffusion of nano-systems will be relatively slower in the crowded cellular environment, an incubation time of 12-hour was selected; this incubation time has been commonly used in many studies about intracellular detections (such as *Anal. Chem.* **2014**, *86*, 12229-12235; *Chem. Sci.*, **2016**, *7*, 1940–1945; *ACS Nano*, **2013**, *7*, 5882-5891; *Nanoscale*, **2015**, *7*, 1753–1759; *J. Am. Chem. Soc*. **2016**, *138*, 306-312; *Sci. Rep.*, **2016**, *6*, 22552.). Therefore, the prolonged incubation time in the cellular experiment may accumulate more signals and increase the sensitivity of the nano-system.

iii. In addition, the instruments for visualizing *in vitro* and cellular detection are different. The cell images are observed by a laser scanning confocal microscope, which uses a laser with high energy to excite the fluorescence dye and will promise a higher resolution for imaging. On the other hand, the *in vitro* detection is characterized by a plate reader with a much lower resolution in collecting fluorescence signals.

iv. Also, the expression of miRNAs in live cell is a dynamic process. Once the miRNAs are hybridized with probes, the miRNA copies available for the formation of RNA-induced silencing complex (RISC) are reduced, therefore, the self-regulation pathway of live cells will be activated by producing more miRNAs from the pre-miRNAs to keep a relative stable number of miRNAs.

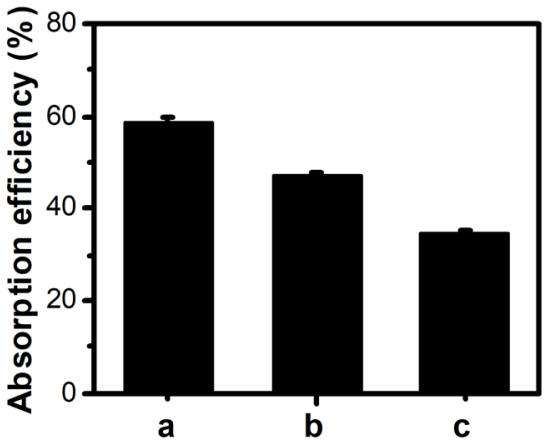

Fig. S2 Physical adsorption efficiency of GO (50 μg/mL) to various DNA probes (1μM). (a) Cy3-probe21, (b) FAM-probe125b, (c) Cy5-probe7a.

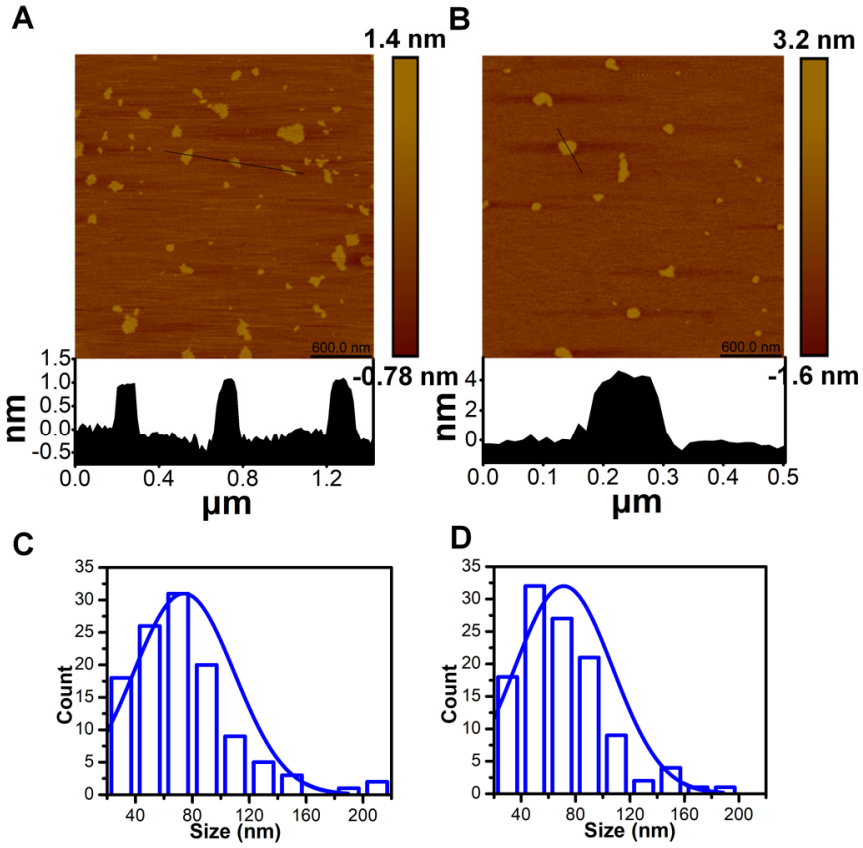

Fig. S3 AFM image and size distribution of pristine GO (A, C) and GBNano-*tri*-whole (B, D).

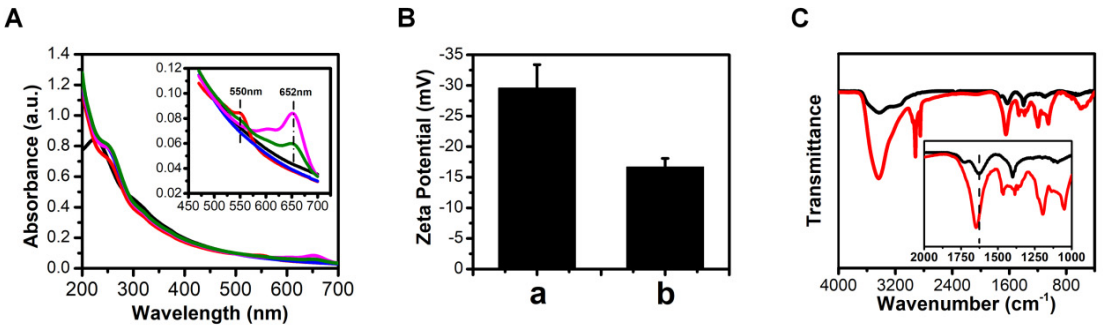

Fig. S4 Characterization of GBNano system. (A) UV-vis spectra of GO (black), GBNano-*mono*-21 (red), GBNano-*mono*-125b (blue), GBNano-*mono*-7a (green) and GBNano-*tri*-whole (pink). (B) Zeta potentials of pristine GO (a) and GBNano-*tri*-whole (b). (C). FT-IR spectra of pristine GO (black) and GBNano-*tri*-whole (red).

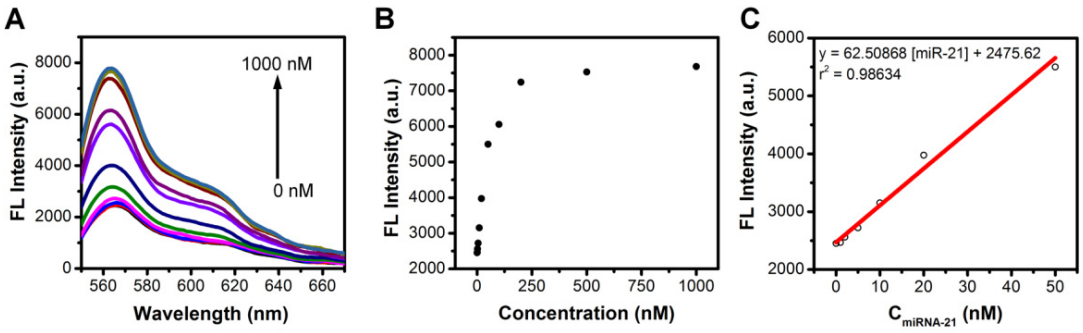

Fig. S5 Extracellular detection of miRNA-21 by FCNano system. The fluorescence spectra (A), calibration curve (B) and liner part of the calibration curve with the concentration of the target miRNA ranging from 0 to 50 nM (C).

We have synthesized the nano-system based on the full-conjugation strategy (which will be called FCNano in abbreviation) and characterized its detection capability for miRNA-21 *in vitro* according to the same protocol used for GBNano. As the concentration of the miRNA-21 was increased from 0 to 1000 nM, the fluorescence of the FCNano increased correspondingly, resulting in a similar F/F_0 ratio compared with that of GBNase. The LOD value is calculated to be 320 pM, which is higher than that of the GBNano nano-system (181 pM). Moreover, the slope of the calibration curve (fitted from a concentration range from 1 to 50 nM) is 62.5, which is much lower than that of the GBNano nano-system (100.2). A lower slope of the calibration curve represents a less obvious change of fluorescence signal (the absolute responsive singal) when the nano-system responds to the target at a certain concentration. Since the grafting efficiency of the FCNano is much lower than that of the GBNano system (as shown in Figure S1 of supporting information), there would be less DNA probes to participate in the detection of target miRNAs, which will result in a lower signal variation. The results indicated that, for miRNA detection, FCNano shows a much lower absolute fluorescence signal compared with GBNano, which will result in a lower sensitivity especially for cellular imaging.

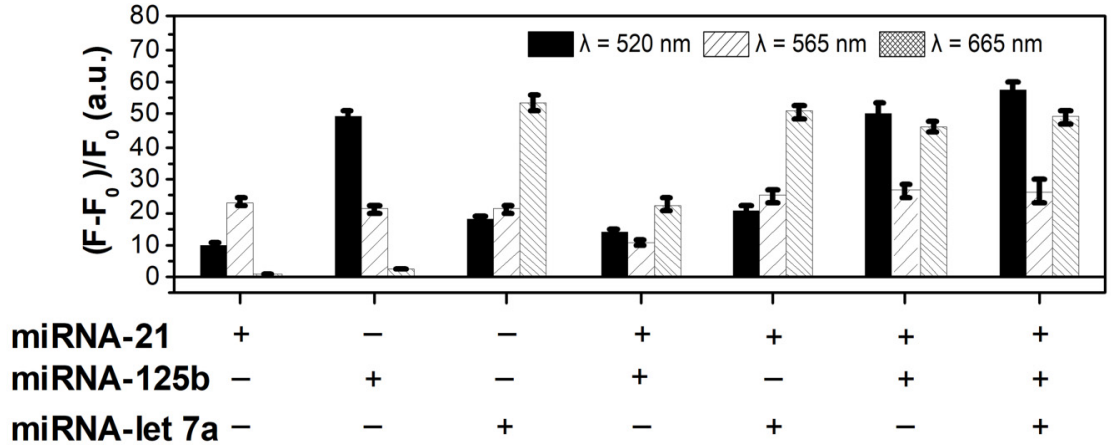

Fig. S6 Specificity and cross-reaction of PNano-*tri*-whole in multiplex detection of miRNAs.

Stability in protein enriched environment. Different concentrations of BSA were added to the HEPES buffer to mimic the high protein environment in serum. After incubation with different concentrations of BSA, the fluorescence of PNano-*mono*-21 intensified gradually as the BSA concentration increased from 0.2 mg/mL to 5 mg/mL (Fig. 2A, red line). When the BSA concentration was 5mg/mL, the fluorescence increased as high as ten times of the GBNano-*mono*-21. That implies that probes could be displaced before uptake by cells, which would decrease the intracellular delivery efficiency.

Stability under DNase I. PNano-*mono*-21 was incubated in the HEPES buffer containing 1U and 3U DNase I (Fig. 2B, red lines). The fluorescence intensified as the incubation time increased. The result demonstrated that the physisorption of GO showed relatively less protection to the DNA probes in the presence of DNase I.

Specificity of multiplex microRNA detection. PNano-*tri*-whole physically adsorbed with

three probes (cy3-probe21, FAM-probe125b and cy5-probe7a) was synthesized and tested, which was incubated with one, two or three kinds of miRNAs (the miRNA concentration is ten times of the corresponding probes). After a 2-h incubation, severe nonspecific signals were observed, regardless of the existence of target miRNAs or not (Fig. S6).

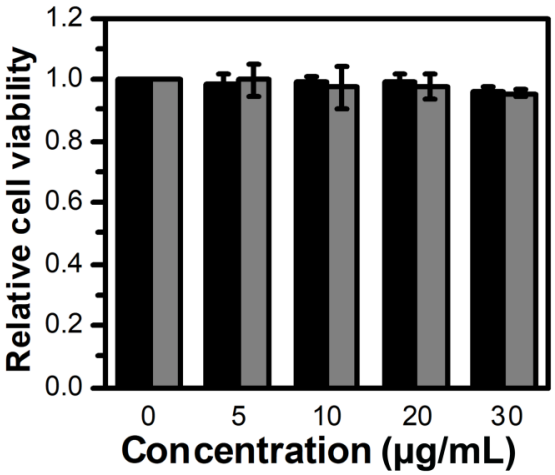

Fig. S7 Cytotoxicity of GBNano-*tri*-whole at different concentrations toward MCF-7 (black) and MCF-10A (gray) cell lines.

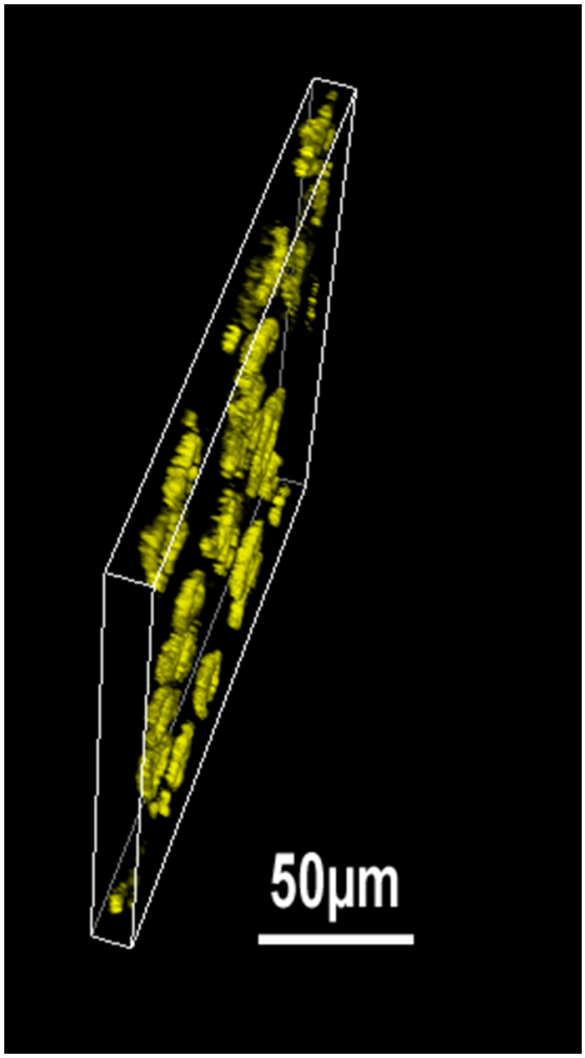

Fig. S8 Three-dimensional LSCM image of MCF-7 cells incubated with GBNano-*mono*-21 for 12h.

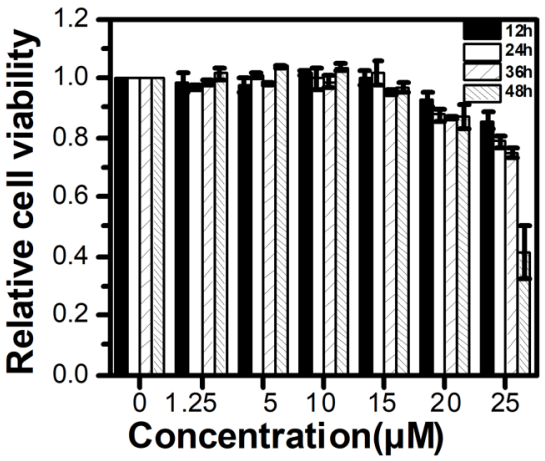

Fig. S9 Cytotoxicity test by the MTT assay for MCF-7 cells incubated with curcumin at different time.

Table S1. Sequence summary

Oligonucleotide name	Sequence with terminal label/modification
Common anchor	5'-NH_2-TCA TCT TTG ACT TTC TCT
Anchor-control	5'-TCA TCT TTG ACT TTC TCT
Cy3-probe21	5'-Cy3-T CAA CAT CAG TCT GAT AAG CTAAGA GAA AGT CAA AGA TGA
FAM-probe125b	5'-FAM-T CAC AAG TTA GGG TCT CAG GGAAGA GAA AGT CAA AGA TGA
Cy5-probe7a	5'-Cy5-A ACT ATA CAA CCT ACT ACC TCAAGA GAA AGT CAA AGA TGA
Cy3-probe-sc	5'-Cy3-A TCG AAT AGT CTG ACT ATG ACTAGA GAA AGT CAA AGA TGA
Cy3-probe21-amine	5'-Cy3-T CAA CAT CAG TCT GAT AAG CTAAGA GAA AGT CAA AGA TGA-NH_2
miRNA-21	5' to 3'UAG CUU AUC AGA CUG AUG UUG A
miRNA-125b	5' to 3' UCC CUG AGA CCC UAA CUU GUG A
miRNA-let 7a	5' to 3' UGA GGU AGU AGG UUG UAU AGU U

Note: (1) The common anchor annealed with DNA probes by the red part to form a partial duplex. (2) The same high light color represents the complementary parts.