A graphene oxide-based platform for the assay of RNA synthesis by RNA polymerase using a fluorescent peptide nucleic acid probe

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Experimental details

Materials
T7 RNA polymerase was purchased from Epicentre Technologies Corporation, Madison, WI, USA. The 94-mer RNA (sequence shown in Fig. S1) was synthesized by in vitro transcription using the T7 RNA polymerase (0.5 μM) in transcription buffer [40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol (DTT)] with 2 mM NTPs (purchased from Genenmed, Seoul, Korea) and DNA template (20 ng/μL, shown in Fig. S1). 6-fluorescein amidite (FAM)-labeled 10-mer PNA and DNA probes (sequence shown in Fig. S1) were custom synthesized and purchased (PANAGENE, Daejeon, Korea, and Cosmogenetech, Seoul, Korea, respectively). Graphene oxide (GO) that was prepared by Hummer's method¹ was purchased from the vendor (cat#. SKU-HCGO-W-175, Graphene Supermarket®, Graphene Laboratories, Inc., Ronkonkoma, NY, USA), and GO-coated mesoporous silica nanoparticle (GO-MSN) was synthesized as described previously.²

Atomic Force Microscopy (AFM) Measurement
The AFM images were collected using an atomic force microscope with NanoScope V controller (Model: Bruker Multimode 8, Bruker AXS Inc., Madison, WI, USA) at room temperature in tapping mode with spring constant of 40 N/m and tip radius of ≤ 8 nm. 10 μL of the graphene oxide solution (10 μg/mL) was used and then was placed on freshly cleaned silicon wafer washed by piranha cleaning method. The sample was dried at room temperature. The scanning speed was at a line frequency of 1.0 Hz, and the original images were sampled at a resolution of 256 × 256 pixels.

Electrophoretic mobility shift assay
FAM-labeled PNA (1 μM) or DNA (1 μM) probe was hybridized with RNA (1 μM) by incubating at 37°C for 20 min in transcription buffer. The same experiment was also performed by mixing DNA (1 μM) with varying amount of RNA (1, 2, and 3 μM). Mixture was then incubated at 37°C for 20 min in transcription buffer. The mixtures were analyzed by 15% non-denaturing polyacrylamide gel electrophoresis (PAGE). Fluorescence images of probes (green color) were obtained by UV illumination, and RNA was identified by staining with ethidium bromide (orange color), followed by UV illumination.

**Fractionation of RNA-PNA hybrid from GO-MSN**

1 μM of PNA was hybridized with various concentrations of RNA (0, 5, and 10 μM) in transcription buffer by incubating at 37°C for 20 min. 5 μL of RNA-PNA hybrid reaction solution was added to a mixture containing 5 μL of 1 mg/mL GO-MSN, 10 μL of 5× transcription buffer, and 30 μL of distilled water. After further incubation at room temperature for 10 min, the mixtures were centrifuged (at 12,470 g for 10 min), and the free RNA-PNA hybrids were obtained by fractionating the supernatant. Pellets containing RNA-PNA hybrid adsorbed on GO-MSN surface were resuspended with distilled water, and the fluorescence intensities of each of the fractions (pellet and supernatant) were measured at an excitation wavelength of 485 nm, and an emission wavelength of 535 nm by using a multilabel plate reader (VICTOR X3; PerkinElmer, Waltham, MA, USA).

**Measurement of the fluorescence emission of PNA probe and RNA-PNA hybrid**

RNA (1 μM) and PNA (1 μM) were hybridized in transcription buffer by incubating at 37°C for 20 min. 50 μL of the RNA-PNA hybrid reaction mixture as added to a solution containing 1, 5, and 10 μL of 50 μg/mL GO-MSN, 100 μL of 5× transcription buffer, and distilled water.
to make up a total volume of 500 μL. After further incubation at room temperature for 10 min, the emission spectra of the mixture were measured using a spectrofluorophotometer (model RF-5301PC; Shimadzu Inc., Kyoto, Japan) in the wavelength range of 500–650 nm upon excitation at 485 nm.

For a multi-well plate experiment, PNA (1 μM) was hybridized with various concentrations of RNA (0–20 μM) in transcription buffer by incubating at 37°C for 20 min. 5 μL of RNA-PNA hybrid reaction solution was mixed with solution containing 1 μL of 50 μg/mL GO-MSN, 10 μL of 5× transcription buffer, and 34 μL of distilled water in 96-well plate. The mixtures were further incubated at room temperature for 10 min, and FAM fluorescence was measured with a multilabel plate reader (VICTOR X3). The excitation wavelength was 485 nm, and the emission wavelength was 535 nm.

**Analysis of RNA synthesis by T7 RNAP**

*In vitro* RNA synthesis was performed in transcription buffer containing 2 mM NTPs, 3 μM PNA, and DNA template (20 ng/μL, shown in Fig. S1). T7 RNA polymerase (1 U/μL, equivalent to 14 nM) was used for RNA synthesis. The reaction mixture was incubated at 37°C for 1 h. Each RNA product was quenched by adding quenching buffer [50 mM Tris (pH 6.8), 0.2% (w/v) SDS, 1 mM EDTA, 10% (w/v) glycerol] with various time points. Each reaction product was analyzed by 10% non-denaturing PAGE. Fluorescence images of probe PNA (green color) were obtained by UV illumination; RNA (orange color) and RNA-PNA hybrid (yellow color) were identified by staining with ethidium bromide (orange color).

For fluorescence-quenching analysis at different concentrations of GO, the *in vitro* transcription mixtures were incubated at 37°C for 60 min, and the 0 min control was prepared before the RNAP addition. The reaction mixtures (5 μL) were directly added to the GO
solution at various concentrations in the transcription buffer with a final volume of 50 µL in a
96-well plate. After 10-min incubation at room temperature, FAM fluorescence was measured
with a multilabel plate reader (VICTOR X3). The excitation wavelength was 485 nm, and the
emission wavelength was 535 nm.

**Fluorometric analysis of RNA synthesis in a 96-well plate**

T7 RNA polymerase (1 U/µL, equivalent to 14 nM) was used for RNA synthesis *in vitro* as
stated above, and 14 nM bovine serum albumin (BSA) was used as a negative control. In
every 10 min, 5 µL of each reaction product was added to a solution containing 3 µL of 50
µg/mL GO (a final concentration of 3 µg/mL), 10 µL of transcription buffer, and 32 µL of
distilled water in a 96-well plate. After further incubation for 10 min at room temperature,
FAM fluorescence was measured using a multilabel plate reader (VICTOR X3). Fluorescence
images of the RNA synthesis reaction in a black 96-well plate (SPL Life Sciences, Gyeonggi-
do, Korea) were obtained using a fluorescent imaging system (IVIS-Lumina II; Caliper
Lifesciences, Hopkinton, MA, USA).

For the RNAP inhibition assay, various concentrations of 3'-deoxy-5-methyluridine
triphosphate (DMUT) were added to the RNA synthesis reaction mixture with 1 U/µL T7
RNAP. After incubating at 37°C for 60 min, 5 µL of each reaction mixture was quenched by
adding solution containing 3 µL of 50 µg/mL GO (a final concentration of 3 µg/mL), 10 µL
of transcription buffer, and 32 µL of distilled water in 96-well plate. The fluorescence
intensities were measured using a multilabel plate reader (VICTOR X3) with an excitation
wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence images in a
black 96-well plate were obtained as above using a fluorescent imaging system (IVIS-Lumina
II).
Comparison of DNA and PNA as a probe in GO-based polymerase assay system

The FAM-labeled 10-mer DNA probe (3 μM) was mixed in transcription buffer containing 2 mM NTPs, and DNA template (20 ng/μL). T7 RNA polymerase (1 U/μL) was then added to the reaction mixture for RNA synthesis. The reaction mixture was incubated at 37 °C for 1 h. RNA synthesis reaction was quenched by adding the quenching buffer at various time points. Each reaction product was analyzed by 15% non-denaturing PAGE.

For comparison of each DNA and PNA probe in the GO-based fluorometric system for RNA synthesis, the in vitro RNA synthesis was performed in transcription buffer containing 2 mM NTPs, and DNA template (20 ng/μL). T7 RNA polymerase (1 U/μL) was used for RNA synthesis and the reaction mixture (50 μL) was incubated at 37°C. In every 10 min, reaction mixture was mixed with a solution containing each probe (PNA or DNA, a final concentration of 0.3 μM). After incubating at 37 °C for 10 min, 5 μL of each annealed product was added to a solution containing 1 μL of 50 μg/mL GO (a final concentration of 1 μg/mL), 10 μL of transcription buffer, and 34 μL of distilled water in a 96-well plate. After further incubation for 10 min at room temperature, FAM fluorescence was measured using a multilabel plate reader (VICTOR X3). Fluorescence images of the RNA synthesis reaction in a black 96-well plate were obtained using a fluorescent imaging system (IVIS-Lumina II).

Radiolabeled method for RNA polymerase assay

In vitro transcription was performed in transcription buffer containing DNA template (20 ng/μL) and T7 RNA polymerase (1 U/μL). Radiolabeled nucleotide [α-32P]UTP (3,000 Ci/mmole, GE Healthcare) was spiked (a final concentration of 2 nM) into the nucleotide mixture containing two different UTP concentrations; 1) 2 mM of all four nucleotides.; 2) 2 mM of ATP, GTP, CTP and 0.2 mM UTP. The reaction mixture was withdrawn and mixed
with the quenching buffer at various time points up to 1 h at 37 °C. The products were resolved by 10% (w/v) denaturing PAGE, and the product bands were visualized and quantified on a Cyclone PhosphorImager (Packard Instruments, Meriden, CT).

References

**Fig. S1.** AFM image with height profile of GO. Three repeated experiments (shown in three different colors) provided the average thickness of 1.176 nm which corresponds to one atomic layer.

**Fig. S2.** Sequences of oligonucleotides; DNA template containing T7 promoter, RNA product, and PNA probe. *In vitro* transcription generates RNA strand occurring at the downstream of T7 promoter. +1 indicates initiation point of RNA synthesis.
Fig. S3. Non-denaturing PAGE (15%) analysis of RNA hybridization with PNA or DNA probes. (a) Stoichiometric amount of RNA and probes (1:1 molar ratio) were incubated at 37 °C for 20 min. (b) Increasing amounts of RNA (molar ratio shown above the gel) were annealed to DNA probe by incubating at 37 °C for 20 min.

For PNA probe, stoichiometric amount of RNA is sufficient for complete annealing whereas DNA probe needs excess amount of RNA to produce annealing product (Free DNA probe still remains in the annealing reaction).
**Fig S4.** (a) Experimental procedure for the fractionation of free RNA-PNA hybrid from GO-MSN-complexed RNA-PNA hybrid. (b) Fluorescence intensities of each fraction with different concentrations of RNA. Supernatant contains free RNA-PNA hybrid and pellet contains the complex of RNA-PNA with GO-MSN. [RNA+PNA] + GO-MSN represents total fluorescence before fractionation.
Fig. S5. (a) Electrophoretic analysis of RNA synthesis by T7 RNAP in the presence of 0.4 μM PNA probe. (b) Shown are the fluorescence images for the detection of RNA-PNA annealing products during \textit{in vitro} transcription at increasing reaction time points. Various concentrations of GO were added to the reaction mixtures containing the PNA probe (0.04 μM).
Fig. S6. (a) Non-denaturing PAGE (15%) analysis of the RNA synthesis using the 10-mer DNA probe. FAM-labeled DNA probes were detected by UV illumination as green fluorescence, and RNA was visualized by staining with ethidium bromide (EtBr). (b) Comparison of the PNA and the DNA probes in the GO-based polymerase assay system. Fluorescence increase was observed with PNA probe in a time-dependent manner, while the DNA probe shows little fluorescence change.
**Fig. S7.** The radiolabeled method for detection of RNA synthesis using [$\alpha$-$^{32}$P]UTP. Unlabeled nucleotides were used in two different conditions; (a) 2 mM NTPs. (b) 2 mM ATP, GTP, CTP, and 0.2 mM UTP.

In the case of the 2 mM NTPs, most of isotope-labeled UTP was mainly unincorporated into RNA with weak product signal (shown in panel a). In contrast, the low concentration of unlabeled UTP provides a better signal for RNA synthesis which was observed by increased RNA band radioactivity (shown in panel b). Thus, a low UTP concentration is necessary for the conventional radiolabeled method, which makes the reaction condition far from the natural condition for RNA synthesis (i.e. same concentrations for all nucleotides).