Supporting Information

for

A Novel Graphene Oxide Amplified Fluorescence Anisotropy assay with Improved Accuracy and Sensitivity

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EXPERIMENTAL SECTION

1. Chemicals and Apparatus

All the DNA oligonucleotides (Table 1) used in the present work were prepared by Sangon Biotech (Shanghai, China). Thrombin, adenosine, guanosine, cytidine, thymidine and uridine were obtained from Sigma Aldrich. Hemoglobin, albumin, amylase, pepsase was purchased from HMK (Beijing, China), Dingguo Changsheng Biotechnology CO., Ltd (Beijing, China), Xiya Reagent (Chengdu, China), Sinopharm Chemical Reagent CO., Ltd (Beijing, China) respectively. GO was purchased from Nanjing XFNANO Materials Tech Inc. (Nanjing, China) and suspended in water via sonication. A 10X TNaK buffered solution was prepared by mixing 200 mM Tris-HCl, 1.4 M NaCl, 200 mM KCl, 100 mM MgCl₂ at pH 7.4. Milli-Q ultrapure water (18.2 MQ) was used for preparing all the solution.

Fluorescence anisotropy (FA) measurement was performed on an F-2500 fluorescence spectrophotometer equipped with a polarization filter (Hitachi, Tokyo, Japan). Solutions were blended using a vortex mixer QL-901 (Haimen, China) before each measurement. A constant-temperature water-base boiler (jiangsu, China) was used for controlling temperature.

2. General procedure

To a mixture, containing cDNA, pDNA and 200 μL ultrapure water, 40 μL 10X TNaK buffered solution was added, and the resulting mixture was incubated at 95 °C for 5 min. The mixture was then cooled to room temperature for 30 min. Next, GO solution (14.0 μg/mL) was added to the above mixture and incubated at room temperature for 60 min. In the final step, target was added to the mixture and the final volume was made to 400 μL by adding ultrapure water. After addition of target, the mixture was allowed to react for 90 min at 37 °C. FA of the reaction mixture was recorded for quantitative analysis.

3. FA Measurements

Fluorescence intensities were measured on F-2500 fluorescence spectrophotometer with an excitation
wavelength of 560 nm, and emission was detected at 581 nm. The FA \( r \) of the test solution was calculated using following equation:

\[
r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}}
\]

and

\[
G = \frac{I_{HV}}{I_{HH}},
\]

where \( I \) is the fluorescence intensity, and the subscripts \( V \) and \( H \) refer to the orientation (vertical or horizontal) of polarizer. The first subscript indicates the position of excitation polarizer, while the second indicates the position of emission polarizer. \( G \) is the instrumental correction factor, which corrects for the different detection efficiencies of parallel and perpendicular emission pathways.

4. Table S1. The list of oligonucleotide sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA1</td>
<td>AAAAAAAAAAAAAAAAAAAAAAAAAACGGGGACAC</td>
</tr>
<tr>
<td>pDNA1</td>
<td>TAMRA - TGTCCTGTGGTCCCCGT</td>
</tr>
<tr>
<td>tDNA</td>
<td>ACGGGGACACCAGACA</td>
</tr>
<tr>
<td>cDNA(N=0)</td>
<td>AAAAAAAAAAAAAAAAAAAAAAAAAACGGGGACACCAGAC A</td>
</tr>
<tr>
<td>cDNA(N=2)</td>
<td>AAAAAAAAAAAAAAAAAAAAAAAAAACGGGGACACCAGA</td>
</tr>
<tr>
<td>cDNA(N=4)</td>
<td>AAAAAAAAAAAAAAAAAAAAAAAAAACGGGGACACCA</td>
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<tr>
<td>cDNA(N=8)</td>
<td>AAAAAAAAAAAAAAAAAAAAAAAAAACGGGGAC</td>
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<tr>
<td>cDNA(N=10)</td>
<td>AAAAAAAAAAAAAAAAAAAAAAACGGGG</td>
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<tr>
<td>t1</td>
<td>ACGGGGACACCACACA</td>
</tr>
<tr>
<td>t2</td>
<td>ACGGGGAGACACCACACA</td>
</tr>
<tr>
<td>t3</td>
<td>ACGGCGGACACCCACAC</td>
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<tr>
<td>nc</td>
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</tr>
<tr>
<td>cDNA2</td>
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</tr>
<tr>
<td>pDNA2</td>
<td>GTACAACTAACCCTGGGGGAGTATTGCGGAGGAAGGT-TAMRA</td>
</tr>
<tr>
<td>cDNA3</td>
<td>AAAAAAAAAAAAAAAAAAAGTTGTACCAACC</td>
</tr>
<tr>
<td>pDNA3</td>
<td>GGTGTTGTTGTTGGGTACAAC</td>
</tr>
</tbody>
</table>
Detection of ssDNA

**Fig. S1** The fluorescence spectra of subnatant after ultrafiltration for ssDNA detection. 1, pDNA1 + cDNA1 + GO + tDNA; 2, pDNA1 + cDNA1 + GO; 3, pDNA1 + GO + tDNA; 4, pDNA1 + GO. Concentrations: cDNA1, 50 nM; pDNA1, 50 nM; GO, 14.0 μg/ml; tDNA, 30 nM.

**Fig. S2** The function of cDNA1 for ssDNA detection. The unidirection fluorescence spectra before (2, 4) and after (1, 3) the addition of tDNA in the presence (1, 2) and absence (3, 4) of cDNA1. Concentrations: cDNA1, 50 nM; pDNA1, 50 nM; GO, 14.0 μg/ml; tDNA, 30 nM. a. HH, b. HV, c. VH, d. VV.
Fig. S3 FA (r) in absence (dark gray columns) and presence (white columns) of tDNA with/without subsequent incubation with cDNA1. Concentrations: cDNA2, 50 nM; pDNA1, 50 nM; GO, 14.0 μg/mL; tDNA, 16 nM. Each measurement was triplicated (error bars indicate standard deviation).

Fig. S4 FA (r) in the absence and presence of the tDNA with different concentrations of GO. The black line, without tDNA; the red line, 30 nM tDNA. Concentrations: cDNA1, 50 nM; pDNA1, 50 nM. Each measurement was triplicated (error bars indicate standard deviation). At concentration of 14 μg/mL of GO, \( \Delta r = r_0 - r \), where \( r_0 \) and \( r \) are the FA values in absence and presence of tDNA, respectively) reached its maximum value. Therefore, 14.0 μg/mL was confirmed as the optimal concentration of GO.
Fig. S5 Optimization of the experimental conditions for ssDNA detection. a, the FA (r) response at different incubation temperature of the complex of cDNA1/pDNA1 on GO. b, the FA (r) response at different incubation time of the complex of cDNA1/pDNA1 with GO. c, the FA (r) response of cDNA1/pDNA1/GO with tDNA at different temperature. d, the FA (r) response of cDNA1/pDNA1/GO with tDNA at different reaction time. Gray columns, in the absence of target DNA; white columns, in the presence of tDNA. Concentrations: cDNA1, 50 nM; pDNA1, 50 nM; GO, 14.0 μg/mL; tDNA, 30 nM. Each measurement was triplicated (error bars indicate standard deviation). The optimized time and temperature are 60 minutes and is room temperature for the incubation of the complex of cDNA1/pDNA1 and GO. On the other hand, the best reaction time and temperature are 90 minutes and 37 °C for the toehold-mediated strand exchange reaction after the addition of tDNA.

Fig. S6 FA (r) in the absence and presence of the tDNA with different base number of toehold domain. Concentrations: cDNA, 50 nM; pDNA1, 50 nM; GO, 14.0 μg/mL; tDNA, 30 nM. Each measurement was triplicated (error bars indicate standard deviation). N is the number of bases of toehold domain.

The experimental results indicate that when the N is small, although the accuracy of the FA measurement could be improved because of the large distance between TAMRA and GO, the pDNA1 could not be able to release from the surface of GO easily, leading to the low Δr. However, when N is too high, background FA is bigger than 0.4 because of the greatly quenched fluorescence intensity of TAMRA on pDNA1 resulted from the short distance between GO and TAMRA. Therefore, the optimal base number of toehold domain was confirmed as 6 for the tDNA detection, wherein the fluorescence quenching effect of GO could be decreased to ensure the accuracy of FA measurement and the pDNA could be released easily from GO surface to ensure the sensitivity for tDNA detection.
Fig. S7 FA (r) responses at different concentrations of tDNA without cDNA1. Concentration: pDNA1, 50 nM; GO, 14.0 μg/mL. Each measurement was triplicated (error bars indicate standard deviations).

Detection of adenosine

Fig. S8 FA (r) in absence (dark gray columns) and presence (white columns) of adenosine with/without subsequent incubation with GO. Concentrations: cDNA2, 50 nM; pDNA2, 50 nM; GO, 14.0 μg/mL; adenosine, 400 μM. Each measurement was triplicated (error bars indicate standard deviations).
Fig. S9 The fluorescence spectra of the subnatant after ultrafiltration for adenosine detection. 1, pDNA2 + cDNA2 + GO + adenosine, 2, pDNA2 + cDNA2 + GO, 3, pDNA2 + GO + adenosine, 4, pDNA2 + GO. Concentrations: cDNA2, 50 nM; pDNA2, 50 nM; GO, 14.0 μg/mL; adenosine, 250 μM.

Fig. S10 Total fluorescence spectra before (2, 4) and after (1, 3) adding adenosine in presence (1, 2) and absence (3, 4) of cDNA2. Concentrations: cDNA2, 50 nM; pDNA2, 50 nM; GO, 14.0 μg/mL; adenosine, 250 μM.
**Fig. S11** The unidirectional fluorescence spectra before (2, 4) and after (1, 3) the addition of adenosine in the presence (1, 2) and absence (3, 4) of cDNA2. Concentrations: cDNA2, 50 nM; pDNA2, 50 nM; GO, 14.0 μg/mL; adenosine, 250 μM. a. HH, b. HV, c. VH, d. VV.

**Fig. S12** FA (r) responses at different concentrations of adenosine without cDNA2. Concentration: pDNA2, 50 nM; GO, 14.0 μg/mL. Each measurement was triplicated (error bars indicate standard deviations).

**Detection of thrombin**

**Scheme S1** A schematic representation of thrombin detection by GO amplified FA assay.
**Fig. S13** The linear relationship between $\Delta r$ and thrombin concentration. Concentration: cDNA3, 50 nM; pDNA3, 50 nM; GO, 14.0 μg/mL. Each measurement was triplicated (error bars indicate standard deviation). The estimated detection limit is as low as 0.19 mg/L (3σ).

**Fig. S14** Selectivity of thrombin detection. Concentrations: cDNA3, 50 nM; pDNA3, 50 nM; GO, 14.0 μg/mL; thrombin, hemoglobin, pepsase, amylase, and albumin, 2.5 mg/L. Each measurement was triplicated (error bars indicate standard deviation).
Fig. S15 FA (r) responses at different concentrations of thrombin without cDNA3. Concentration: pDNA3, 50 nM; GO, 14.0 μg/mL. Each measurement was triplicated (error bars indicate standard deviation).