Fluorescence Imaging of Intracellular Telomerase Activity Using Enzyme-Free Signal Amplification

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Supporting Information

ABSTRACT: A novel enzyme-free signal amplification-based assay for highly sensitive in situ fluorescence imaging and detection of intracellular telomerase activity was developed by using a gold nanoflare probe-triggered mimic-hybridization chain reaction (mimic-HCR) coupled with a graphene oxide (GO) surface-anchored fluorescence signal readout pathway. The nanoflare probe consists of gold nanoparticles (AuNPs) functionalized with a dense shell of nucleic acid sequences by Au–S bond formation. The nucleic acid sequence is composed of three segments: a long thiol-labeled sequence (HS-DNA) and two short sequences (a telomerase primer sequence, “Primer-DNA”, and an FAM-terminated reporter sequence, “Flare-DNA”), both of which are complementary to HS-DNA. The mimic-HCR system is formed by two FAM-modified hairpin sequences that are adsorbed on GO. Upon endocytosis of the AuNP/GO combinatorial probe, the Primer-DNA can be extended by intracellular telomerase at its 3′ end to produce the telomeric repeated sequence, which leads to inner chain substitution and not only releases the Flare-DNA to turn on the fluorescence of FAM but also initiates the subsequent signal amplification and enrichment for the mimic-HCR system anchored on GO. The proposed approach can sensitively detect telomerase activity in living cells, distinguish normal cells from cancer cells, and monitor the change in telomerase activity in response to a telomerase inhibitor.

Telomerase is a ribonucleoprotein complex that adds the TTAGGG hexamer repeats at chromosome ends to ensure the indefinite proliferation of cells.† Substantial studies reveal that more than 85% of cancer cells are positive for telomerase, but most of normal somatic cells are negative. Accordingly, telomerase can be considered not only as a diagnostic and prognostic biomarker but also as a potential target for managing cancer.‡ So it is significant to determine the cell-to-cell variation in telomerase activity for different samples.³ However, in humans, the abundance of telomerase is only approximately one hundred molecules per cell, even in a telomerase-positive tumor cell.⁵ Most of common methods for detecting telomerase activity are useful for the bulk samples that usually were obtained by lysing multiple cells, such as the real-time telomere repeat amplification protocol (RT-TRAP),⁶ the exponential isothermal amplification of telomere repeat (EXPIATR) assay,⁶ catalytic beacons,⁷ surface plasmon resonance,⁸ fluorescence,⁹ electrochemistry,¹⁰ and electrochemiluminescence.¹¹ But these assay methods are incapable of detecting the relative telomerase level in individual cell. Therefore, the ability to analyze the cell-to-cell variation of telomerase activity is essential for achieving real-time monitoring of intracellular events.

As we know, molecular imaging can provide in situ information on biological indicators or biomarkers of cancer.¹² Through in situ fluorescence imaging, it can reveal the intracellular activity of telomerase and monitoring the change in cellular telomerase activity in response to telomerase-related drugs. Recently, Ju’s group has reported several noninvasive methods for the in situ detection of intracellular telomerase.¹³⁻¹⁵ However, either the preparation was complicated for the telomerase-responsive mesoporous silica nanoparticle probe and the liposome-based vesicle kit or the probe displayed a certain degree of nonspecific release and environmental susceptibility.¹⁵ Therefore, the development of robust and more sensitive methods, for example, signal amplification-based protocol, for in situ tracking of intracellular telomerase activity is still of great importance. Overall, most of signal amplification-based methods are highly enzyme-dependent, in which at least one type of enzyme is necessary to catalyze the amplification process.¹³,¹⁵ Therefore, the reaction conditions such as temperature, pH, and the environmental media must be controlled to ensure the enzymes, which greatly limits their applications for in situ analysis of targets in living cells. Monitoring targets of interest in live cells via enzyme-free
amplification, which enables visualization of low-expression targets with high signal gain in live cells and avoids damaging or even killing cells by delivering exogenous enzymes or proteins, is attracting increasing attention from researchers. The Tan group reported a nonenzymatic hairpin DNA cascade reaction that provides high signal gain for mRNA imaging inside live cells. Our group recently introduced an enzyme-free amplification strategy to image low-level microRNA in living cells using the hybridization chain reaction (HCR) with graphene oxide (GO) as the carrier.

In this work, we proposed an intracellular enzyme-free signal amplification telomerase detection method, based on the combination of an AuNP-based nanoflare probe (“AuNP probe”) and a GO-loaded mimic-HCR system (“AuNP GO probe”; see Scheme 1). Previously the Mirkin group has reported the concept of the nanoflare to develop an fluorophore-labeled oligonucleotide duplexes AuNP conjugate that was capable of both detecting and regulating intracellular mRNA levels. Similarly, in the present nanoflare probe, the HS-DNA/Primer-DNA/Flare-DNA (“hybridized-DNA”) was bound to AuNPs by the Au-S bonds and the terminal fluorophore FAM of the Flare-DNA was quenched via fluorescence resonance energy transfer (FRET). Pierce and coauthor introduced the concept of HCR, in which stable DNA hairpin sequences (H1-DNA and H2-DNA, respectively), which were designed to alternate hybridize to generate the nicked double-stranded DNA structures just as HCR upon the introduction of initiator (Trigger-DNA), but the leakage of the system will occur. Additionally, the H1+H2 system was absorbed on GO by π-π stacking, and the fluorescence of the FAM labels was quenched by GO. DNA-modified AuNPs and GO can directly enter cells by endocytosis without any transfection agent. After the AuNP/GO probe enters into cancer cells, Flare-DNA dissociates from the AuNPs upon Primer-DNA extension, inspired by intracellular telomerase. The existence of telomerase can be demonstrated by the fluorescent signals. Furthermore, released Flare-DNA can efficiently initiate GO-loaded mimic-HCR between two species of fluorescent hairpin sequences (H1 and H2) for a signal amplification and enrichment role, and this proposed method has avoided the effect of exogenous enzymes on in situ analysis of telomerase in living cells. Thus, the AuNP/GO probe can be conveniently used for highly sensitive in situ imaging and detection of intracellular telomerase activity through a one-step incubation procedure.

Scheme 1. Schematic Illustration of the AuNP-Based Nanoflare Probe (AuNP Probe, A) and AuNP and GO-Based Combined Probe (AuNP/GO probe, B) for In Situ Analysis of Intracellular Telomerase

**EXPERIMENTAL SECTION**

**Preparation of Hybridized-DNA Functionalized AuNP Probe.** First, HS-DNA (100 μL, 1 OD) was treated by TCEP (1.5 μL, 100 mM) for 1 h. The activated HS-DNA, Flare-DNA (28 μL, 100 μM), and Primer-DNA (28 μL, 100 μM) was mixed, heated to 75 °C, and incubated for 10 min. Then the mixture was slowly cooled to room temperature and placed in the dark for at least 12 h to allow complete hybridization. Then the obtained hybridized-DNA was mixed with 2 mL of AuNPs. After incubating overnight at room temperature, 0.1 mL of PBS solution containing 2 M NaCl was added to the mixture stepwise for stabilizing the probe. A total of 24 h later, the mixture was purified by centrifuging for 30 min at 13500 rpm and washed with EXPIATR buffer twice to remove excess DNA. The AuNP probes were finally redispersed in 1 mL of EXPIATR buffer and stored at 4 °C for further use.

**Telomerase Response Experiment.** To demonstrate the release of Flare-DNA form AuNP probe in response to telomerase, the fluorescence intensity of the mixture of AuNP probe (160 μL, 2 nM), dNTPs (20 μL, 10 mM each) in the presence or absence of commercial telomerase standard (20 μL, 20 IU·L⁻¹) was detected after different incubation times at 37 °C. Under excitation at 488 nm, the fluorescence of FAM was recorded from 490 to 600 nm.

**Polyacrylamide Hydrogel Electrophoresis Experiments of Telomerase-Triggered Flare-DNA Releasing.** Six different samples were injected into polyacrylamide hydrogel in tris-borate-EDTA (TBE) buffer. Six lanes from 1 to 6 are listed as (Lane 1) 2 μL of Flare-DNA (100 μM) and 18 μL of telomerase diluents; (Lane 2) 5 μL of Primer-DNA (100 μM) and 15 μL of telomerase diluents; (Lane 3) 5 μL of HS-DNA (100 μM) and 15 μL of telomerase diluents; and (Lane 4) the mixture of 5 μL of Flare-DNA (100 μM), 5 μL of HS-DNA (100 μM), 5 μL of Primer-DNA (100 μM), and 35 μL of telomerase (20 IU·L⁻¹) were held at 37 °C for 2 h; (Lane 5) the mixture of 5 μL of Flare-DNA (100 μM), 5 μL of HS-DNA (100 μM), 5 μL of Primer-DNA (100 μM), and 10 μL of dNTPs (10 mM), and 10 μL of telomerase (20 IU·L⁻¹) were held at 37 °C for 2 h; and (Lane 6) 10 μL of marker DNA (10 μM) as indicator in 1.5 μL of loading buffer. Electrophoresis was
carried out at 120 V in TBE buffer for 1 h. The resulting board was observed under UV irradiation. The gel was prepared by 8 mL of polyacrylamide (30%), 7 µL of TEMED (86 nM), 4 mL of 0.5x tris-borate-EDTA (TBE), and 0.14 mL of ammonium persulfate (10%).

Preparation of Mimic-HCR/GO System and Trigger-DNA Triggered Fluorescence Recovery Effect for the Mimic-HCR/GO System. First, the FAM-labeled hairpin DNA probes (H1 and H2) were separately heated at 75 °C for 10 min and then allowed to cool to room temperature for 1 h before use. Subsequently, GO (the final concentration is 25 µg·mL⁻¹) was added into the system, and the final mixture was further incubated for 12 h to obtain the mimic-HCR/GO probe. Then, in a final volume of 100 µL of reaction solution, different concentrations of Trigger-DNA were incubated with the above system for 4 h at 37 °C to proceed the mimic-HCR. For the fluorescence imaging of the mimic-HCR/GO system, the final reaction mixture was separately dropped onto a 20 mm confocal dish, and the fluorescence imaging test was performed on a FV-1200 laser-scanning fluorescence confocal microscope (Olympus) by using a 488 nm laser.

Fluorescent Detection of Telomerase Activity in Cell Extract with Two Protocols. A standard telomerase extension reaction mixture (200 µL) contains variable amounts of cell extract, dNTPs (0.5 mM each), and AuNP probe (1.5 nM) or AuNP/GO probe (AuNPs 1.5 nM and GO 25 µg·mL⁻¹). After fully stirring, the resulting solution was held at 37 °C for 2 h. Under excitation at 488 nm, the fluorescence intensity of the mixture was detected from 490 to 600 nm.

In Situ Imaging of Telomerase Activity in HeLa, A549, MCF-7, Caco-2, HBL-100, or HL-7702 Cells, or Drug-Treated HeLa Cells. After human cervical cancer cells (HeLa; 0.5 mL, 1 × 10⁶ mL⁻¹) were seeded in a 20 mm confocal dish for 24 h, AuNP probe (1.5 nM), AuNP/GO probe (AuNPs 1.5 nM and GO 25 µg·mL⁻¹) or H1+H2/GO system (GO 25 µg·mL⁻¹) was added into each cell-adhered dish. After incubation at 37 °C for 2 h, the cells were sent for fluorescent confocal imaging.

Similarly, after A549, MCF-7, Caco-2, HBL-100, or HL-7702 cells (0.5 mL, 1 × 10⁶ mL⁻¹) were seeded in a 20 mm confocal dish for 24 h, AuNP probe (1.5 nM), AuNP/GO probe (AuNPs 1.5 nM and GO 25 µg·mL⁻¹) was added into each cell-adhered dish. After incubation at 37 °C for 2 h, the cells were sent for fluorescent confocal imaging.

After HeLa cells (0.5 mL, 1 × 10⁶ mL⁻¹) were seeded in a 20 mm confocal dish for 24 h, 60, 120, 240, or 400 µg·mL⁻¹ EGCG were added into each adhered dish. Later, AuNP/GO probe (AuNPs 1.5 nM and GO 25 µg·mL⁻¹) was added, respectively, to the four dishes mentioned above and incubated for 2 h. Then the cells were sent for fluorescent confocal imaging. But after the above process, cells treated with 400 µg·mL⁻¹ EGCG floated on the liquid surface (defined as the state of death) and were not suitable for confocal imaging.

RESULTS AND DISCUSSION

Characterization of Nanoprobes. After the AuNPs were functionalized with the DNA, the UV–vis spectrum still showed the typical AuNPs peak at 520 nm with a slight shift (Figure S1B-b). But the hydrodynamic size of AuNPs determined by DLS changed obviously. As shown in Figure S1C,D, the average size of 13.5 ± 0.3 nm for the bare AuNPs increased to 38.7 ± 1.9 nm for AuNP probe. The increase of the hydrodynamic size is in accordance with 2-fold of the length of the HS-DNA. The amount of the hybridized-DNA assembled on each AuNP (about 120 hybridized-DNA per nanoparticle) was determined by the mercaptoethanol competing experiment, which confirmed the successful binding of the hybridized-DNA to AuNPs (Figure S2). Obviously, the surface coverage here is higher than the value (90 ± 10) reported by Mirkin. This phenomenon should be caused by the specific three components structure of the hybridized-DNA, which show the higher rigidity than the duplex structure designed by Mirkin which has a long flexible single-strand segment at the end of the HS-DNA.

In Vitro Studies of Telomerase Activity with Nanoprobes. The principle of the detection of telomerase activity with the AuNP probe is schematically shown in Scheme 1A. The fluorescence of the Flare-DNA loaded on AuNPs was in the “turned-off” state because FAM was quenched by a FRET mechanism. The observed fluorescence signal indicated the release of Flare-DNA, providing important information about the telomerase. To demonstrate the release of Flare-DNA from the AuNP probe in response to telomerase, an amount of AuNP probes were incubated with commercial telomerase and dNTPs for 2 h under optimized conditions (see Supporting Information), and the incubated solution was subjected to fluorescence analysis. As shown in Figure 1A, almost no fluorescence was detected for FAM in the absence of telomerase (curve a), suggesting that the FAM fluorescence was quenched by the AuNPs. The fluorescence intensity was significantly increased after the addition of telomerase (curve b), indicating the release of Flare-DNA from the AuNP probe due to competition with the extension product of the Primer-DNA. In addition, a gradual increase of fluorescence intensity with an increasing incubation time was also observed (curves b–n), and the fluorescent signal reached the maximum value after 60 min (Figure S3). Importantly, no change in the fluorescence intensity was observed in the absence of telomerase in 2 h (Figure S3, inset, curve a), verifying the good stability of the AuNP probe and the telomerase-triggered Flare-DNA release mechanism.

The mechanism of switching via telomerase-triggered Primer-DNA elongation was verified by gel electrophoresis (Figure 1B). After incubation of the Flare-DNA, Primer-DNA, and HS-DNA for 2 h, the product was approximately 70 bp (Figure 1B, Lane 4-a), which was the same as the total of all three sequences mentioned above (Figure 1B, Lanes 1–3), confirming the formation of the hybridized-DNA. Additionally, there was a trace amount of unhybridized Flare-DNA. After the

Figure 1. (A) Fluorescence spectra of the AuNP probe and dNTPs in the absence (a) or presence (b) of telomerase. (B) Polyacrylamide gel electrophoresis image. Lane 1: Flare-DNA; Lane 2: Primer-DNA; Lane 3: HS-DNA; Lane 4: the hybridized-DNA and dNTPs after incubation for 2 h; Lane 5: the hybridized-DNA, dNTPs and telomerase after incubation for 2 h; Lane 6: marker DNA.
hybridized-DNA was mixed with dNTPs and telomerase solution and incubated for 2 h. Lane 5 also displayed a band at approximately 70 bp with relatively low intensity, but the Lane 5-d band was more intense than the Lane 4-b band, demonstrating the extension of the inner Primer-DNA in the hybridized-DNA triggered by telomerase with the concomitant increase in the signal characteristic of Flare-DNA below 15 bp. From the position of the extension product of the hybridized-DNA (Figure 1B, Lane 5c), it can be seen that approximately 2 TTAGGG segments were extended for the Primer-DNA, which is consistent with the previous report that at 37 °C mostly shorter products are produced (∼1–3 repeats).3

With the nanoflare detection system, only one fluorophore lights up with one target interaction (Scheme 1A), thus, offering limited sensitivity. To improve the sensitivity, a GO-based mimic-HCR method was proposed, in which a single target could trigger the lighting-up of several fluorophores of the excess FAM-labeled hairpin sequences absorbed on GO.

The principle of the mimic-HCR amplification telomerase detection with the AuNP/GO probe is schematically shown in Scheme 1B. To test the protocol, this process was characterized by gel electrophoresis (Figure S8). After a period of reaction, for the mixture of H1 (Lane 2) and H2 (which has the same number of nucleotides as H1, so it should be found in the similar place to H1, not shown here), the majority existed as H1 and H2 monomers, but small amounts were copolymerized with each other to yield large molecular weight DNA complexes shown in the upper part of the lane (Lane 3). The leakage phenomenon of H1 and H2 in the absence of Flare-DNA should be resulted from the special segments of TTAGGG, which has also been reported in a recent study using a mimic-HCR-based telomerase assay.7 Once Flare-DNA was added into the system of H1 and H2, a HCR event can be triggered and the longer double helix increased (Lanes 4 and 5). When the Flare-DNA was increased to a suitable amount, the unreacted species almost disappeared completely and formed a longer double helix, indicating the completion of the mimic-HCR process (Lane 6).

In addition, the fluorescence recovery of the Trigger-DNA (with the same sequence as the Flare-DNA but not modified by FAM)-initiated mimic-HCR/GO system was confirmed by fluorescence imaging (Figure S9). These results demonstrate that the Trigger-DNA-induced fluorescence signals of the mimic-HCR/GO system are dominantly located on the GO surface through π−π stacking of the mimic-HCR product terminal single-strand sequence and GO. This phenomenon is very favorable to the signals enrichment shown by the fluorescence imaging and the increased in situ detection sensitivity. Additionally, the Flare-DNA-triggered mimic-HCR/GO system resulted in an approximately 2-fold increase in the fluorescence intensity compared with the Flare-DNA alone (Figure S10).

According to the present design, the amount of released Flare-DNA reflects the telomerization of the Primer-DNA and is consistent with the concentration of telomerase in the samples. Correspondingly, when cell extracts from different numbers of cells were used as the telomerase source, the detection sensitivity of the present two strategies can be obtained by determining the intensity of the fluorescence signal. With AuNP probe alone, the capability of the strategy shown in Scheme 1A for telomerase detection is investigated. As shown in Figure 2A, under the optimized conditions, gradual enhancement of the fluorescence intensity was observed with increasing the cell number after the incubation of the AuNP probe with dNTP and cell lysates which come from the serially diluting of the cell extracts from 1.0 × 10^6 HeLa cells. Notably, the fluorescence intensity exhibited a linear correlation with the cell number over a range from 2000 to 40000 cells (Figure 2A, inset). The regression equation is Y = 28.911 + 3.0725X with a correlation coefficient of 0.9987, where Y is the fluorescence intensity and X is the cell number. The limit of detection is 480 cells based on the calculation of the average signal of the blank plus three times the standard deviation. According to the reported value of telomerase activity in one HeLa cell,13 3.1 × 10^-11 IU, the detection limit of this method is 1.488 × 10^-8 IU.

Next, with the system containing the AuNP/GO probe, dNTP, and cell lysate we investigated the signal amplification ability of the mimic-HCR process in real samples. As shown in Figure 2B, with the mimic-HCR process the fluorescence intensity was also enhanced when analyzing the telomerase extracts from the gradually increasing number of HeLa cells from 2000 to 40000. However, with the same number of HeLa cells, the signal amplification system had higher fluorescence intensity than the AuNP probe system. This method can measure telomerase activity down to 220 HeLa cells (∼1 cell/μL) based on the calculation of the average signal of the blank plus three times the standard deviation, and the corresponding detection limit of telomerase activity is equal to 6.82 × 10^-9 IU. With HeLa cells as the research object, the detection limit of this work and some other methods for telomerase analysis were summarized in the Supporting Information (see Table S2). Comparatively, the detection limit of this work is close to that of the reported DNAzyme-based fluorescence assay,5,10,29 but is higher than those of the enzyme-based amplification assays.5,10,29

In Situ Imaging of Intracellular Telomerase Activity. For in situ imaging of intracellular telomerase activity, HeLa cells were separately incubated with the AuNP probes, AuNP/GO probes, or H1+H2/GO system for 2 h under the optimized conditions (see Supporting Information) and were then analyzed by confocal laser scanning microscopy (CLSM). In the presence of telomerase in cell extracts, the optical probes were “turned on” by a telomerase-induced DNA competing mechanism, as mentioned above. Similarly, upon reaching the target cancer cells, the FAM-modified optical imaging sequences of Flare-DNA were released from the AuNPs, resulting in the fluorescence being “turned on”. As shown in Figure 3A,B, fluorescence signals were clearly observed, and
Figure 3B, derived from the AuNP/GO amplification system, shows stronger fluorescence intensity than Figure 3A, which is based only on AuNP probes. No fluorescence was observed after the cells were incubated with H1+H2/GO (Figure 3C). These results indicate that two types of nanoprobes were successfully transported into the cells and that the mimic-HCR was triggered in the living cells, leading to a higher fluorescence signal than that obtained via only Au-nanoflare fluorescence. For obtaining the best confocal images, the optimization of the concentration of AuNP probe and the mimic-HCR system was shown in the Supporting Information.

We then used two types of probes to track the telomerase activity in three different cancer cell lines (Figure 4), including human lung cancer cells (A549), human breast cancer cells (MCF-7), and human colon cancer cells (Caco-2). Confocal images revealed that although the AuNP probe can be used to monitor the telomerase activity of various cancer cell types, the AuNP/GO probe has a more sensitive fluorescence imaging capability, especially for MCF-7 cells, which show lower telomerase activity. But due to the low telomerase activity in normal human breast cells (HBL-100) and human liver cells (HL-7702), although with the AuNP/GO probe confocal images can not give obvious Flare-DNA related fluorescent response (Figure S11). So we can see that the present assays can be used to distinguish normal cells from cancer cells.

Figure 4. Confocal images of A549, MCF-7, and Caco-2 cells after incubation with different probes: (A, C) AuNP probe and (B, D) AuNP/GO probe; fluorescence field (A, B), and overlapped fluorescence and bright-field images (C, D).

Protective Properties of Probe to DNA Against Nuclease, MTT Assay, and Stability of AuNP/GO Probe.

To investigate the protective properties of AuNP/GO probe against damage by intracellular enzymes, DNase I was added to the AuNP/GO probes in phosphate-buffered saline (PBS) or EXPIATR buffer, and almost no fluorescence signal enhancement was observed compared with the background (Figure S12). The cytotoxicity of the AuNP/GO probe was examined by the MTT assay. The HeLa cells maintained approximately 92.3% cell viability after incubation with the probe for 3 h (Figure S13), indicating satisfactory low cytotoxicity. In addition, the stability of AuNP/GO composite probe was studied. As Figure S14 shown that no obvious aggregate was observed after AuNP probe and the mimic-HCR probe were mixed, and this state can maintain for more than 12 h.

Monitoring the Change of Telomerase Activity in Response to Inhibitor.

The AuNP/GO probe was further employed for in situ monitoring of the change of the intracellular telomerase activity after treatment with different concentrations of epigallocatechin gallate (EGCG), a well-characterized telomerase inhibitor. As shown in Figure 5, the fluorescence intensity gradually decreased with increasing amounts of EGCG, confirming that the AuNP/GO probe were specific to telomerase activity. Additionally, the...
fluorescence intensity of the same concentration of EGCG-treated HeLa cells was higher than that in other studies. This phenomenon is due to the further signal amplification by the mimic-HCR system, which was particularly obvious for the cells treated with the highest concentration of EGCG, at which the local fluorescence signal distributed in the cytoplasm near the nucleus could still be observed (Figure 5C,F), but the fluorescence completely disappeared in the studies that used mesoporous silica nanoparticle probes or nicked molecular beacon-functionalized AuNP probes.

Conclusion

In conclusion, a novel enzyme-free signal amplification protocol was successfully presented for the highly sensitive in situ detection of intracellular telomerase activity. The feasibility of this protocol was confirmed by using HeLa cells as the telomerase-positive sample, and the dose-dependent change in telomerase activity in response to the telomerase inhibitor was monitored directly. Given the ability of AuNPs and GO to act as gene-loading agents, these probes may be easily adapted to simultaneously transfect and visualize gene expression in real time, and this enzyme-free signal amplification method can be extended to the in situ detection and imaging of other intracellular biomolecules using DNA hybridization technology.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03108. Part of experimental details and supporting figures mentioned in the main text (PDF).

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Notes

The authors declare no competing financial interest.

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