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PAPER

Multiplex detection of nucleases by a graphene-based platform†

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In this article, we present a new method for the multiplex detection of nucleases by using graphene oxide (GO) as a platform. We introduce a Y-shaped DNA (Y-DNA) as the multiplex probe. The 5′ termini of the Y-DNA are labeled with carboxy fluorescein (FAM), 6-carboxy-X-rhodamine (ROX) and cyanine 5 (Cy5) and they include three nuclease cleavage sites corresponding to PvuII, EcoRV and HaeIII, respectively. Upon the addition of nucleases, the nucleases cleave the corresponding sites in Y-DNA. Then, short dsDNA fragments containing fluorophores were released from the Y-DNA. These dsDNA fragments were unstable and easy to unwind into two short ssDNAs. They were then adsorbed onto the GO surface. Because of the excellent electronic transference of GO, the fluorescence intensity of the fluorophores can be quenched efficiently. Therefore, by monitoring the fluorophores' fluorescence change before and after the addition of the nucleases, it is easy to establish a platform of a Y-DNA/GO complex for the simultaneous multiplex detection of nucleases.

Introduction

Restriction endonucleases, well-known nucleases that strictly recognize and hydrolyze specific sequences on double-stranded DNA or RNA, are among the most important enzymes in molecular biology.[1,2] The specific actions of these enzymes are essential in PCR assays, gene mapping, molecular cloning and medicinal chemistry.[3–6] The development of sensitive and selective techniques capable of assaying enzyme activities has become a great task in the fields of clinical diagnostics, drug discovery and nanoscience. The typical assay methods for nuclease activity studies include gel electrophoresis, capillary isoelectric focusing, high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA).[7–10] However, these methods are laborious, insensitive, time-consuming, require sophisticated instruments and usually require radiolabeling of the substrates. To address these problems, fluorescence assays based on fluorescence quenching or fluorescence resonance energy transfer (FRET)[11–14] have been developed to monitor nuclease activity. For example, molecular break lights or molecular beacons have been developed for the highly sensitive and convenient assay of restriction endonucleases, using a single-stranded oligonucleotide as the substrate.[11,12,15] However, these fluorescence methods need double fluorophore-labeled DNA probes. Furthermore, these methods have not been used for the multiplex detection of nucleases simultaneously. In this respect, Wang's group has developed a new design for the multiplex detection of endonucleases by using a cationic conjugated polymer.[16] Although this method is convenient for the simultaneously multiplex detection of nucleases, the synthesis of the cationic conjugated polymer required a relatively complex procedure. Therefore, there is still a need for other methods of detecting multiplex nucleases in one system.

In this work, we present a platform for the multiplex detection of nucleases by using the property of graphene oxide (GO) to preferentially bind single-stranded DNA (ssDNA) over double-strand DNA (dsDNA). The efficient adsorption of ssDNA on the GO surface is likely due to hydrophobic and π-stacking interactions between the nucleobases and graphene.[17–19] However, dsDNA cannot be stably adsorbed on the GO surface because of the efficient shielding of nucleobases within the negatively charged phosphate backbone of the dsDNA.[20] Up to now, many scientists have focused on exploring graphene in the analysis of biomolecules.[20–27] In this article, we introduce a Y-shaped DNA (Y-DNA) as the multiplex probe. Y-DNA pioneered by Luo's group consists of three complementary oligonucleotide branches and paves the way for the multiplex detection of DNA.[28,29]

Fig. 1 shows a schematic representation of this new detection platform. A GO sheet is used as both the "nanoscaffold" for the oligonucleotide and the "nanoquencher" for the fluorophores. Each of the 5′ termini of the Y-DNA are labeled individually with carboxy fluorescein (FAM), 6-carboxy-X-rhodamine (ROX) and cyanine 5 (Cy5), and include nuclease cleavage sites corresponding to the enzymes PvuII, EcoRV, and HaeIII,

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† Electronic supplementary information (ESI) available: DNA sequences, XRD patterns, FT-IR spectra and kinetic behavior. See DOI: 10.1039/c1jm11121c

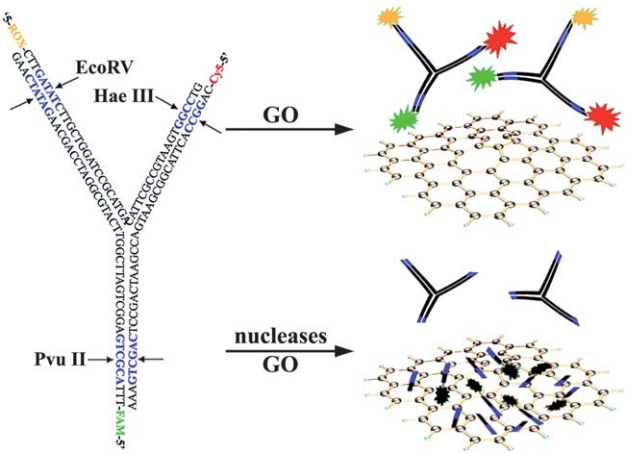

Fig. 1 A GO-based platform for the multiplex detection of nucleases (DNA sequences are shown in the electronic supporting information†).

respectively. Since Y-DNA cannot be stably adsorbed onto the GO surface, we anticipate that Y-DNA may maintain the fluorescence intensities in the presence of GO. Upon the addition of a nuclease, the nuclease can cleave the corresponding site in the Y-DNA. Then, a short dsDNA fragment containing a fluorophore is released from the Y-DNA. This short dsDNA fragment is unstable and easy to unwind into two short ssDNAs. It is then adsorbed onto the GO surface. Due to the excellent electronic transference of GO, the fluorescence intensity of the fluorophore could be quenched efficiently. Meanwhile, the fluorophore attached to the intact dsDNA will maintain the fluorescence intensity. Therefore, we can use Y-DNA and GO for the multiplex detection of nucleases.

Experimental

2.1. Materials

Oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co. (China). The HaeIII, PvuII, and EcoRV endonucleases were purchased from New England Biolabs Inc. (Beverly, MA, USA).

2.2. Preparation of graphene oxide

Graphene oxide (GO) was synthesized from natural graphite powder by a modified Hummers method.[30] Briefly, graphite powder (2 g) was ground with NaCl to reduce the particle size. After removal of the salt, the graphite was added to concentrated H_2SO_4 (80 mL) and left stirring for 2 h. Afterwards, $KMnO_4$ (10 g) was gradually added with stirring and the temperature of the mixture was kept at less than 20 °C. The mixture was then stirred at 35 °C for 2 h. Keeping the temperature at less than 50 °C, distilled water (180 mL) was added and then the mixture was stirred at room temperature for 3 h. The reaction was ended by a final addition of a distilled water (450 mL) and H_2O_2 (30%, 20 mL) solution. Finally, the mixture was repeatedly washed with a 1 : 10 HCl aqueous solution and then with distilled water. Exfoliation was carried out by sonicating the graphene oxide (2 mg mL^{-1}) dispersion under ambient conditions for 4 h.

2.3. Kinetic behavior of the Y-DNA/GO complex toward PvuII

0.5 U of PvuII was added to a solution of total volume 20 μL containing Y-DNA (2 μL, [Y-DNA] = 10 μM), and this mixture was reacted over time at 37 °C. After that, the solution was diluted with 0.5 mL Tris-HCl buffer (20 mM, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$). GO (6 μg mL^{-1}) was immediately added to the solution, and the fluorescence spectra were measured at room temperature with an excitation wavelength of 485 nm.

2.4. Assays of DNA cleavage by nucleases

The restriction nucleases (0.5 U PvuII, 1.0 U EcoRV, 0.5 U HaeIII) were added to a solution of total volume 20 μL containing Y-DNA (2 μL, [Y-DNA] = 10 μM). After incubation at 37 °C for 0.5 h, the solution was diluted with 0.5 mL Tris-HCl buffer (20 mM, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$). After the addition of GO (6 μg mL^{-1}), the fluorescence spectra were measured at room temperature with an excitation wavelength of 485 nm for the FAM fluorescence spectrum, 580 nm for the ROX fluorescence spectrum and 630 nm for the Cy5 fluorescence spectrum.

2.5. Assays of PvuII activity

Different concentrations of PvuII (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 25 U/mL) were added to a solution of total volume 20 μL containing Y-DNA (2 μL, [Y-DNA] = 10 μM). After incubation at 37 °C for 0.5 h, the solution was diluted with 0.5 mL Tris-HCl buffer. After addition of GO (6 μg mL^{-1}), the fluorescence spectra were measured at room temperature with an excitation wavelength of 485 nm.

Results and discussion

3.1. Nondenaturing polyacrylamide gel

We used a nondenaturing polyacrylamide gel to investigate the viability of our strategy. Nondenaturing polyacrylamide gels can be used to analyze dsDNA. As shown in Fig. 2, the untreated Y-DNA itself moves as a single band (lane 1). As envisioned, upon addition of one of the three nucleases, the cleavage site of

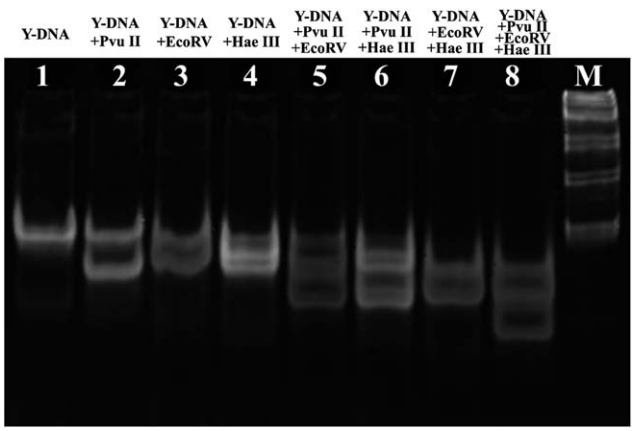

Fig. 2 Nondenaturing polyacrylamide gel (12%) analysis of Y-DNA cleavage by restriction nucleases (M = marker).

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the Y-DNA is cleaved with the corresponding nuclease and the number of Y-DNA base pairs decreases. Therefore, we can see a new faster-moving band (lanes 2–4). Furthermore, when the Y-DNA is simultaneously treated with two or three nucleases, the Y-DNA produces additional DNA fragments. As shown in Fig. 2, the faster-moving bands correspond to the residue of the Y-DNA (lanes 5–8).

3.2. Characterization of GO

Atomic force microscopy (AFM) is the most direct method of quantifying the degree of exfoliation to a single graphene sheet level after the dispersion of the powder in water. Fig. 3 shows a typical AFM image of the prepared GO. According to the cross-section analysis, the thickness of the prepared GO is about 1.2 nm and this matches well with the reported oxide thickness of single-sheet graphene.[31] Fig. S1 (ESI†) shows the XRD patterns of the GO and graphite. The pattern of the GO reveals a sharp 002 reflection at $2\theta = 10.3°$. The most intense peak occurs for graphite at $2\theta = 26.4°$. After oxidation, the interlayer space of the GO is larger than that of the graphite, as a result of the introduction of oxygenated functional groups onto the carbon sheets. As shown in Fig. S2 (ESI†), the FT-IR spectrum gives the characteristic vibrations of GO and graphite, and this matches well with that reported previously.[32] The characteristic vibrations of GO are a broad and intense peak of the O–H group at 3400 cm^{-1}, a C=O peak at 1730 cm^{-1}, a C–OH stretching peak at 1220 cm^{-1}, an C–O stretching peak at 1050 cm^{-1} and a peak attributed to the vibrations of unoxidized graphitic skeletal domains and the adsorbed water molecules at 1620 cm^{-1}. As for graphite, the spectrum is essentially featureless except for the C=C conjugation at 1590 cm^{-1} and O–H group at 3409 cm^{-1}.

3.3. Optimization of GO concentration

In this work, we initially optimized the concentration of GO. Due to the kinetic behavior of the Y-DNA/GO complex toward PvuII, we know that the nuclease cleavage of DNA reaches equilibrium in 0.5 h (Fig. S3, ESI†). We took PvuII as an example (when treated with PvuII, the Y-DNA was cleaved and it released the FAM-labeled DNA fragment). 0.5 U of PvuII was added to a solution of total volume 20 μL containing Y-DNA (2 μL, [Y-DNA] = 10 μM). After incubation at 37 °C for 0.5 h, the solution was diluted with 0.5 mL Tris-HCl buffer. Then, different concentrations of GO were added and the fluorescence spectra were measured at room temperature. As shown in Fig. 4a, when the concentration of GO is higher than 6 μg mL^{-1}, the fluorescence intensity of FAM was almost quenched completely. This means that most of the cleaved DNA fragments were adsorbed onto the GO surface. We also used untreated Y-DNA to compare the efficiency of the fluorescence quenching. We found that only a small decrease in the fluorescence intensity can be observed (Fig. 4b). This result demonstrates that GO associates only weakly with Y-DNA. Therefore, 6 μg mL^{-1} of GO was chosen for the multiplex detection of nucleases.

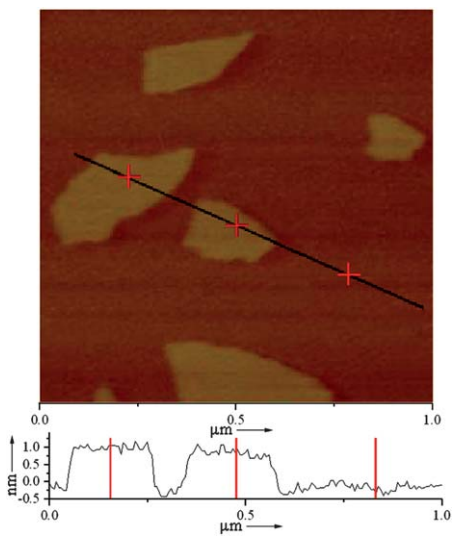

Fig. 3 An AFM image of the GO sheets. A droplet of the GO dispersion (about 0.01 mg mL^{-1}) was cast onto a freshly cleaved mica surface, followed by drying at room temperature.

3.4. Assays of one nuclease

Fig. 5 demonstrates the ability of the Y-DNA/GO complex to detect a single nuclease. Upon adding PvuII, a DNA fragment carrying FAM was generated. This DNA fragment was adsorbed onto the GO surface and the fluorescence intensity of FAM was quenched by GO. At the same time, the residual Y-DNA could not be adsorbed onto the GO surface, so the high fluorescence intensity of ROX and Cy5 can still be observed (Fig. 5a). The Y-DNA/GO complex can also be used for the detection of EcoRV or HaeIII, in a similar way to the detection of PvuII. When Y-DNA was cleaved by EcoRV, only ROX was quenched by the GO (Fig. 5b). For HaeIII, only the emission intensity of Cy5 was reduced with GO (Fig. 5c). Therefore, we can use the Y-DNA/GO complex to detect a single nuclease by monitoring the fluorescence signal change of the corresponding fluorophore.

3.5. Assays of two nucleases

The Y-DNA/GO complex can also be used to detect two nucleases simultaneously. Upon the simultaneous addition of

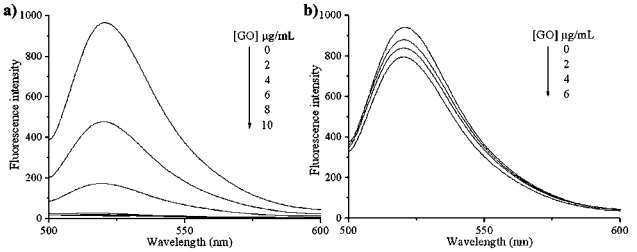

Fig. 4 Fluorescence quenching was performed with various concentrations of GO. a) Y-DNA was treated with PvuII. The fluorescence intensity was quenched quickly when GO was added. b) Untreated Y-DNA was measured for comparison of the quenching efficiency. The fluorescence spectra were obtained on a Hitachi F-4600 spectrofluorometer.

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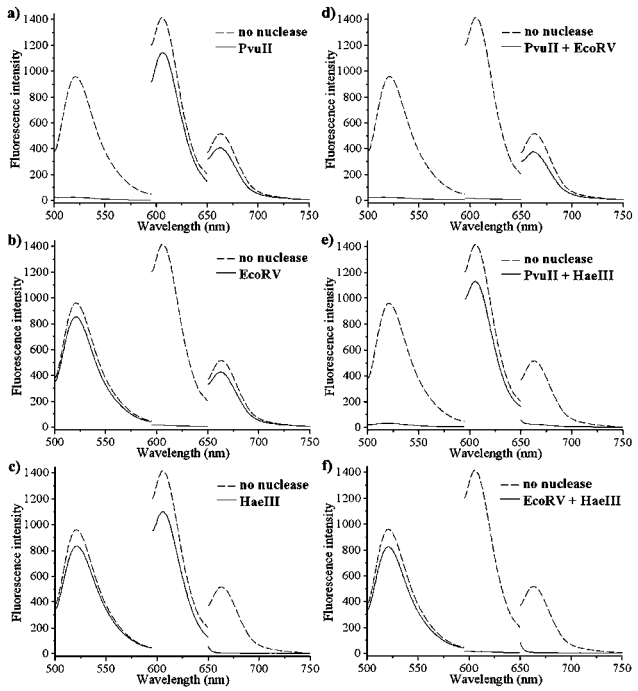

Fig. 5 Fluorescence emission spectra of the Y-DNA/GO complex after Y-DNA was treated with one of the three nucleases (a–c) and after treatment with two of the three nucleases (d–f). Amounts of nucleases: 0.5 U PvuII, 1.0 U EcoRV, 0.5 U HaeIII. The fluorescence spectra of the three dyes in the Y-DNA were measured and then these three spectra were combined into a single figure. The measurement conditions of FAM were excitation at 485 nm, excitation and emission slits of 5 nm and 10 nm and a PMT voltage of 700 V. The measurement conditions of ROX were excitation at 580 nm, excitation and emission slits of 10 nm each and a PMT voltage of 700 V. The measurement conditions of Cy5 were excitation at 630 nm, excitation and emission slits of 10 nm each and a PMT voltage of 900 V.

PvuII and EcoRV, DNA fragments carrying FAM and ROX were generated. Both of these fluorophores were adsorbed onto the GO surface and the fluorescence intensities were quenched by the GO. At the same time, the fluorescence intensity of Cy5 did not change greatly (Fig. 5d). Also, when the Y-DNA/GO complex was treated with PvuII and HaeIII, the fluorescence intensities of FAM and Cy5 were quenched by the GO (Fig. 5e). When the Y-DNA/GO complex was treated with EcoRV and HaeIII, only the fluorescence of FAM can be observed (Fig. 5f). Therefore, the Y-DNA/GO complex can be used to simultaneously detect PvuII & EcoRV, PvuII & HaeIII and EcoRV & HaeIII pairs.

3.6. Assays of three nucleases

The Y-DNA/GO complex can also be used to detect the three nucleases simultaneously. When the Y-DNA was mixed with the three nucleases, the DNA fragments carrying FAM, ROX or Cy5 were released from Y-DNA. These fragments were adsorbed onto the GO surface, resulting in fluorescence intensity quenching of these three fluorophores (Fig. 6). Therefore, by monitoring the fluorophores' fluorescence change before and after the addition of the nucleases, it is easy to establish a platform of the

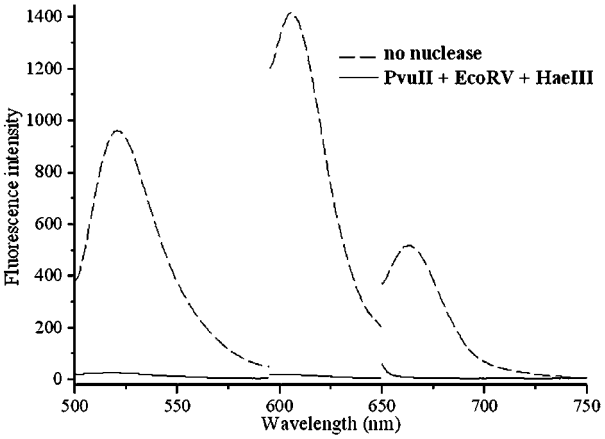

Fig. 6 Fluorescence emission spectra of the Y-DNA/GO complex after the Y-DNA was treated with the three nucleases simultaneously (0.5 U PvuII, 1.0 U EcoRV, 0.5 U HaeIII), with excitation wavelengths of 485 nm (FAM), 580 nm (ROX) and 630 nm (Cy5). The measurement conditions of the three dyes in Y-DNA were the same as those described in Fig. 5.

Y-DNA/GO complex for the simultaneous detection of three nucleases.

3.7. Assays of PvuII activity

Furthermore, the Y-DNA/GO complex can be used to detect the amount of nuclease. Here, we take PVU II as an example. Fig. 7 shows the fluorescence intensity changes upon the addition of different concentrations of PvuII (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 25 U mL^{-1}). The fluorescence intensity of FAM is gradually decreased by increasing the concentration of PvuII. The detection limit of PvuII was 0.05 U mL^{-1}. Therefore, this assay is convenient, quick, sensitive and has a wide dynamic range, making it a useful method for analyzing restriction endonucleases.

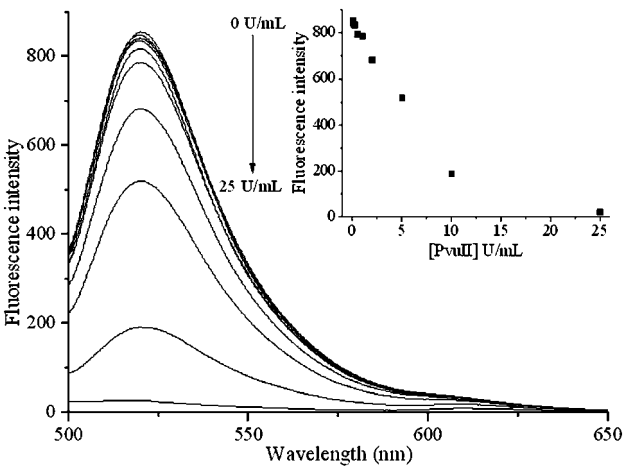

Fig. 7 Fluorescence emission spectra of FAM of the Y-DNA in the presence of different concentrations of PvuII and GO (6 μg mL^{-1}). Excitation: 485 nm, emission: 520 nm.

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Conclusions

In summary, we have developed a new platform for the multiplex detection of nucleases. To the best of our knowledge, this work is the first to utilize GO for a multiplex nuclease assay. The GO-based platform has several advantages over conventional methods. Firstly, this new platform allows for the simultaneous multiplex detection of nucleases by observation of the fluorescence signals of different fluorophores. Secondly, the nuclease activity can be monitored in real time by following the change in the fluorescence. Thirdly, the GO-based assay is cost effective compared to conventional assays. Furthermore, this method has great potential for the high-throughput screening of nucleases and their inhibitors. This work is under way now in our laboratory and will be communicated in due course.

Acknowledgements

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Notes and references

1 E. S. Rangarajan and V. Shankar, *FEMS Microbiol. Rev.*, 2001, **25**, 583.
2 A. Pingoud and A. Jeltsch, *Nucleic Acids Res.*, 2001, **29**, 3705.
3 M. Ma, L. Benimetskaya, I. Ebedeva, J. Dignam, G. Takle and C. A. Stein, *Nat. Biotechnol.*, 2000, **18**, 58.
4 M. R. Lieber, *BioEssays*, 1997, **19**, 233.
5 T. M. Marti and O. Fleck, *Cell. Mol. Life Sci.*, 2004, **61**, 336.
6 N. D. Grindley, K. L. Whiteson and P. A. Rice, *Annu. Rev. Biochem.*, 2006, **75**, 567.
7 K. Shimura, H. Matsumoto and K. Kasai, *Electrophoresis*, 1998, **19**, 2296.
8 S. Spitzer and F. Eckstein, *Nucleic Acids Res.*, 1988, **16**, 11691.
9 K. Hiramatsu, H. Miura, S. Kamei, K. Iwasaki and M. Kawakita, *J. Biochem.*, 1998, **124**, 231.
10 S. P. Lee and M. K. Han, *Methods Enzymol.*, 1997, **278**, 343.
11 J. B. Biggins, J. R. Prudent, D. J. Marshall, M. Ruppen and J. S. Thorson, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 13537.
12 J. Li, H. F. Yan, K. M. Wang, W. H. Tan and X. W. Zhou, *Anal. Chem.*, 2007, **79**, 1050.
13 J. J. Li, R. Geyer and W. H. Tan, *Nucleic Acids Res.*, 2000, **28**, E52.
14 K. Eisenschmidt, T. Lanio, A. Jeltsch and A. Pingoud, *J. Biotechnol.*, 2002, **96**, 185.
15 U. Kettling, A. Koltermann, P. Schwille and M. Eigen, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 1416.
16 X. L. Feng, X. R. Duan, L. B. Liu, F. D. Feng, S. Wang, Y. L. Li and D. B. Zhu, *Angew. Chem., Int. Ed.*, 2009, **48**, 5316.
17 H. Jang, Y. K. Kim, H. M. Kwon, W. S. Yeo, D. E. Kim and D. H. Min, *Angew. Chem., Int. Ed.*, 2010, **49**, 5703.
18 Z. Liu, J. T. Robinson, X. M. Sun and H. J. Dai, *J. Am. Chem. Soc.*, 2008, **130**, 10876.
19 N. Varghese, U. Mogera, A. Govindaraj, A. Das, P. K. Maiti, A. K. Sood and C. N. R. Rao, *ChemPhysChem*, 2009, **10**, 206.
20 S. J. He, B. Song, D. Li, C. F. Zhu, W. P. Qi, Y. Q. Wen, L. H. Wang, S. P. Song, H. P. Fang and C. H. Fan, *Adv. Funct. Mater.*, 2010, **20**, 453.
21 C. H. Lu, H. H. Yang, C. L. Zhu, X. Chen and G. N. Chen, *Angew. Chem., Int. Ed.*, 2009, **48**, 4785.
22 Y. Wang, Z. H. Li, D. H. Hu, C. T. Lin, J. H. Li and Y. H. Lin, *J. Am. Chem. Soc.*, 2010, **132**, 9274.
23 C. H. Lu, J. Li, M. H. Lin, Y. W. Wang, H. H. Yang, X. Chen and G. N. Chen, *Angew. Chem., Int. Ed.*, 2010, **49**, 8454.
24 H. F. Dong, W. C. Gao, F. Yan, H. X. Ji and H. X. Ju, *Anal. Chem.*, 2010, **82**, 5511.
25 M. Zhang, B. C. Yin, X. F. Wang and B. C. Ye, *Chem. Commun.*, 2011, **47**, 2399.
26 W. Yang, K. R. Ratinac, S. P. Ringer, P. Thordarson, J. J. Gooding and F. Braet, *Angew. Chem., Int. Ed.*, 2010, **49**, 2114.
27 K. P. Loh, Q. L. Bao, G. Eda and M. Chhowalla, *Nat. Chem.*, 2010, **2**, 1015.
28 Y. Li, Y. T. Hong and D. Luo, *Nat. Biotechnol.*, 2005, **23**, 885.
29 Y. Li, Y. D. Tseng, S. Y. Kwon, L. D'Espaux, J. S. Bunch, P. L. McEuen and D. Luo, *Nat. Mater.*, 2004, **3**, 38.
30 W. S. Hummers and R. E. Offeman, *J. Am. Chem. Soc.*, 1958, **80**, 1339.
31 X. B. Fan, W. C. Peng, Y. Li, X. Y. Li, S. L. Wang, G. L. Zhang and F. B. Zhang, *Adv. Mater.*, 2008, **20**, 4490.
32 J. F. Shen, Y. Z. Hu, M. Shi, X. Lu, C. Qina, C. Li and M. G. Ye, *Chem. Mater.*, 2009, **21**, 3514.

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