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Increasing the Sensitivity and Single-Base Mismatch Selectivity of the Molecular Beacon Using Graphene Oxide as the "Nanoquencher"

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Abstract: Here, we report a novel, highly sensitive, selective and economical molecular beacon using graphene oxide as the "nanoquencher". This novel molecular beacon system contains a hairpin-structured fluorophore-labeled oligonucleotide and a graphene oxide sheet. The strong interaction between hairpin-structured oligonucleotide and graphene oxide keep them in close proximity, facilitating the fluorescence quenching of the fluorophore by graphene oxide. In the presence of a complementary target DNA, the binding between hairpin-structured oligonucleotide and target DNA will disturb the interaction between hairpin-structured oligonucleotide and graphene oxide, and release the oligonucleotide from graphene oxide, resulting in restoration of fluorophore fluorescence. In the present study, we show that this novel graphene oxide quenched molecular beacon can be used to detect target DNA with higher sensitivity and single-base mismatch selectivity compared to the conventional molecular beacon.

Keywords: biosensors · DNA recognition · graphene · molecular beacons · single-base mismatch

Introduction

The sequence-specific detection of DNA plays a significant role in many areas, such as molecular diagnostics, environmental monitoring, and antibioterrorism. Consequently, various optical and electrochemical techniques have been employed for the development of DNA sensors.[1–3] Among them, molecular beacons (MBs) have been reported to have high specificity in the detection of nucleic acid sequences.[4] MBs are hairpin-shaped DNA molecules with a self-complementary stem that brings a terminal-labeled fluorophore and a quencher into close proximity, causing the fluorescence of the fluorophore to be quenched by energy transfer. When an MB hybridizes with its complementary target, the beacon undergoes a spontaneous conformational reorganization with the opening of the stem, leading to a fluorescence restoration.[5] Although the unique structural and thermodynamic properties of MBs have led to their widespread use in real-time monitoring of DNA/RNA amplification reactions[6,7] and visualization of RNA expression in living cells,[8–11] they still have some drawbacks. First, the sensitivity of conventional MBs is limited due to incomplete quenching of the fluorophore. Second, synthesis of conventional MBs by using a fluorophore and a quenching moiety is usually tedious and costly. Third, the single-base-mismatch selectivity of conventional MBs is not high enough for genetic mutation analysis. Consequently, it is still highly desirable to develop new, sensitive, specific, cost-effective molecular beacons for DNA detection.

Recently, molecular beacons using nanomaterials as "nanoquenchers" have attracted considerable attention. Dubertret and co-workers have developed DNA functionalized gold nanoparticles to achieve efficient quenching and single-base mismatch detection by replacing dabcyl with 1.4 nm gold clusters (nanogold).[12] Under favorable conditions, the fluorescence quenching efficiency can be up to 99.96%, and the ability to detect single-base mismatches is eightfold greater with this gold-quenched MB than with conventional MBs. However, the tedious processes of the preparation of the gold nanoparticles and covalent labeling of the DNA with the nanoparticles limit gold-quenched MB application in bioanalysis. More recently, Tan and co-workers used

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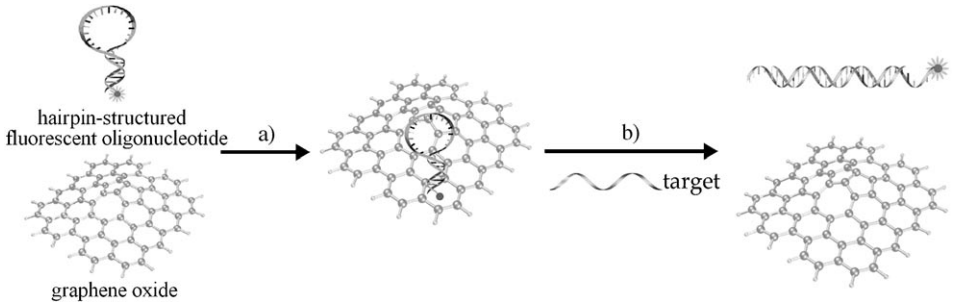
single-walled carbon nanotubes (SWNTs) as the "nanoquencher" for MBs.[13] SWNTs also have high fluorescence quenching efficiency and thus greatly improve the signal-to-background ratio compared with those for conventional MBs.

Graphene, a single layer of carbon atoms in a closely packed honeycomb two-dimensional structure, is a new kind of carbon nanostructure material, which was first produced in 2004.[14] It has attracted great attention because of its remarkable electronic, mechanical, and thermal properties.[15] Graphene has been exploited in many applications, such as composites,[16] Li ion batteries,[17] and electrochemical biosensors.[18,19] Recently, we have demonstrated the ability of water soluble graphene oxide (GO) as a platform for highly sensitive and selective detection of DNA and proteins.[20]

Here, we present a novel highly sensitive, selective and economical molecular beacon biosensor using soluble graphene oxide (GO) as the "nanoquencher" (Scheme 1). The



Scheme 1. Schematic representation of target-induced fluorescence change of GO-quenched molecular beacon (see text for details).

key components of the novel GO-quenched beacon are the hairpin-structured fluorophore-labeled oligonucleotide and GO sheet, which functions as both "nanoscaffold" for the oligonucleotide and "nanoquencher" for the fluorophore. Upon addition of a complementary DNA strand to the system, the hairpin-structured oligonucleotide hybridizes to form a double-stranded DNA molecule, resulting in the release of the oligonucleotide from GO and the fluorescence restoration of the fluorophore. In contrast to the conventional MBs, the novel GO-quenched MB needs only one labeled fluorophore. This design not only provides a more convenient one-step synthesis/purification protocol compared to conventional MBs, but also improves sensitivity due to the exceptional high fluorescence quenching efficiency of GO. Furthermore, the use of GO as the "nanoquencher" can greatly improve the single-base mismatch selectivity of an MB because of the conformational constraint of hairpin-structured oligonucleotides on GO surfaces.

Results and Discussion

Characterization of GO: GO was synthesized from natural graphite powder by a modified Hummers method.[21] Atomic force microscopy (AFM) has been the most direct method of quantifying the degree of exfoliation to a single graphene sheet level after the dispersion of the powder in water. A droplet of graphene oxide dispersion (about 0.01 mg mL^{-1}) was cast onto a freshly cleaved mica surface and dried at room temperature. Figure 1 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 matched well with the reported oxide thickness of single-sheet graphene.[22]

Choice of oligonucleotide sequence and target DNA: Here, a hairpin (HP)-structured oligonucleotide containing a 15-base loop and a 5-mer stem was chosen as the recognition element (Table 1). P1 is a conventional molecular beacon, which was designed by attaching fluorescein (FAM) and 4-[4′-(dimethylamino)phenylazo] benzoic acid (dabcyl)[23] to the 3' and 5' ends, respectively. P2 was used to examine the effect of GO as the "nanoquencher" and was only labeled with FAM at the 3' end in the HP structure. The target single-stranded DNA molecules T1 and T2 were 15 bases long. T1 was perfectly complementary (pc) to the bases in the loop of P1 and P2, while T2 contained a single-base mismatch (sm) within the loop. T1 is from the sequence of the surviving transcript, a target that has received

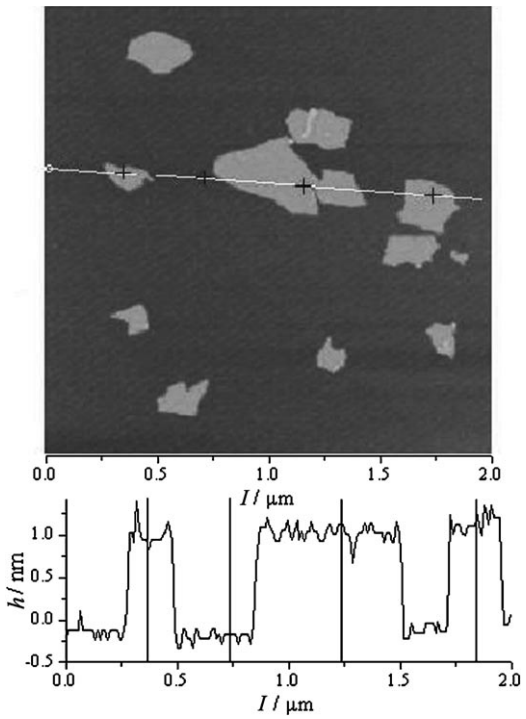


Figure 1. AFM height image of GO sheets deposited on mica substrates.

Table 1. Sequences of the oligonucleotides and target DNA used.

Type	Sequence
FAM-labeled MB (P1)[a]	5′-dabcyl-**CGACG**GAGAAAGGGCTGCCA**CGTCG**-FAM-3′
FAM-labeled HP (P2)[b]	5′-**CGACG**GAGAAAGGGCTGCCA**CGTCG**-FAM-3′
Cy5-labeled MB (P3)[a]	5′-dabcyl-**CGACG**GAGAAAGGGCTGCCA**CGTCG**-Cy5-3′
Cy5-labeled HP (P4)[b]	5′-**CGACG**GAGAAAGGGCTGCCA**CGTCG**-Cy5-3′
pc-DNA (T1)[c]	5′-TGGCAGCCCTTTCTC-3′
sm-DNA (T2)[d]	5′-TGGCAGCGCTTTCTC-3′

[a] Molecular beacon; [b] hairpin-structured probe; [c] perfectly complementary target; [d] single-base-mismatched target (the mismatched base is underlined).

significant attention due to its potential use in cancer therapeutics and diagnostics.[24]

In order to prove the generality of the study, we also used another fluorescent dye (short cyanine Cy5) for analysis. P3 is a conventional molecular beacon with Cy5 and dabcyl at the 3' and 5' ends, respectively. P4 was labeled only with Cy5 at the 3' end in the HP structure.

Fluorescence quenching efficiency of GO: To prove the potential use of GO as "nanoscaffold" and "nanoquencher" for MBs, we investigated the fluorescence emission change of FAM-labeled hairpin-structured oligonucleotide P2 caused by GO. As showed in Figure 2A, P2 has a strong fluorescence emission owing to the presence of the fluorescein-based dye (curve a). However, in the presence of GO, up to 99.1% quenching of the fluorescence was observed (Figure 2A, curve b). It should be mentioned that when using dabcyl, only a quenching efficiency of about 92.9% was observed. This observation indicates the strong adsorption of the HP-structured oligonucleotide on GO and the exceptional high fluorescence quenching efficiency of GO. According to previous studies, this efficient absorption of HP-structured oligonucleotides is likely due to hydrophobic and π-stacking interactions between the nucleobases and graphene.[25–27] In order to illustrate the generality of the method, we also investigated the fluorescence emission change of Cy5-labeled hairpin-structured oligonucleotide P4 caused by GO. A quenching efficiency of about 99.3% was observed (Figure 2B). These results demonstrate that GO can efficiently quench various fluorescent dyes over a wide wavelength range. The exceptional high fluorescence quenching efficiency of GO might derive from the excellent electronic transference of graphene.

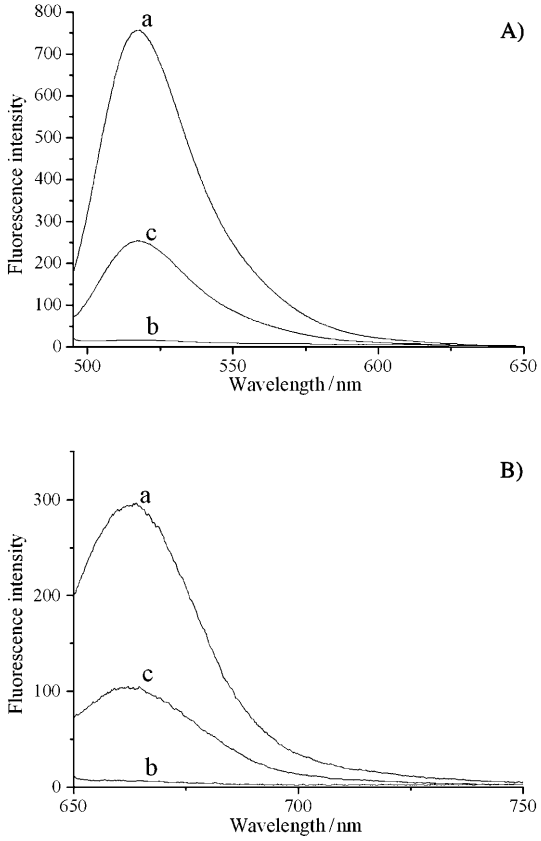


Figure 2. Changes in the fluorescence emissions of: A) P2, and B) P4 caused by GO. Fluorescence emission spectra of P2 and P4 in the absence (curve a) and presence (curve b) of GO, and after addition of a fivefold excess of T1 in the presence of GO (curve c). The concentrations of P2 and P4 were 50 nM.

DNA detection sensitivity of GO-quenched MBs: To demonstrate the potential use of GO-quenched MBs in the detection of target DNA, we investigated their fluorescence restoration in the presence of target DNA. A significant enhancement in fluorescence was observed after addition of fivefold excess of pc-DNA target T1 to P2–GO (Figure 2A, curve c). This indicated that the target DNA can hybridize with the HP-structured oligonucleotide P2 on the GO surface and release P2 from GO resulting in restoration of fluorescence. To compare the sensitivity of GO-quenched MBs with conventional MBs, we employed signal-to-background ratio (S/B) to reflect fluorescence restoration of different MBs. The S/B was defined as $(F_{hybrid}-F_{buffer})/(F_{MB}-F_{buffer})$, where F_{buffer}, F_{MB}, and F_{hybrid} are the fluorescence intensities of the plain buffer solution, the MB without target, and the MB–target hybrid, respectively.[13] As shown in Figure 3, the strong background fluorescence of P1 and P3 led to lower S/B ratios. In contrast, since the exceptional high fluorescence quenching efficiency of GO can decrease background fluorescence intensity, the S/B ratios of P2–GO and P4–GO generated by addition of T1 are significantly improved compared to P1 and P3. This comparison clearly demonstrates that GO can greatly improve the S/B, and consequently, the sensitivities of MBs.

Figure 4 shows the fluorescence intensity changes of P2–GO upon addition of different concentrations of T1. A dramatic increase in FAM fluorescence intensity was observed as the target DNA concentration was increased from 5 to 500 nM. The detection limit (taken to be three times the standard deviation in the blank solution) was 2.0 nM, which

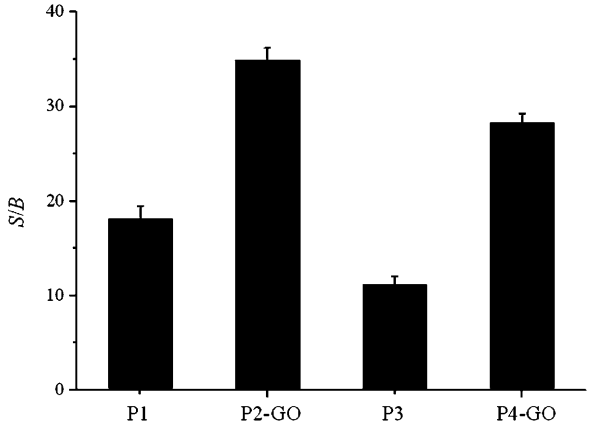


Figure 3. Comparisons of the signal-to-background ratio (S/B) of the MBs generated by a fivefold excess of the pc-DNA target T1. The concentrations of P1, P2, P3 and P4 were 50 nM. The columns represent mean values of three measurements.

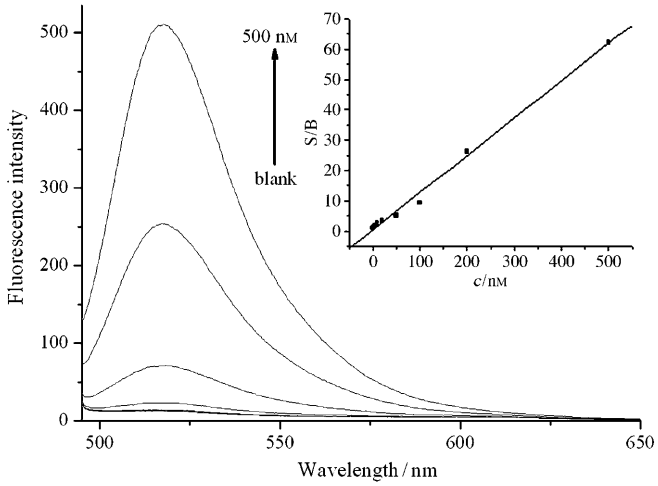


Figure 4. Fluorescence emission spectra of P2–GO (50 nM) in the presence of different concentrations of T1. Inset: signal-to-background ratio (S/B) plotted against the concentration of T1.

is 16-fold lower than that of the conventional MB, P1. The significantly improved S/B and sensitivity compared to conventional MBs will help future biomedical applications of GO-quenched MBs.

DNA detection selectivity of GO-quenched MBs: One of the significant advantages of MBs is that they have high selectivity with which they can be used to detect target sequences. In this study, we compared the single-base mismatch selectivity of GO-quenched MB and a conventional MB. Two MBs were used: one is the conventional MB (P1) and the other is the GO-quenched MB (P2–GO). Using either of the two MBs we were able to distinguish between the perfect target and the single-base mismatch one, but the selectivity differs significantly. The selectivity described herein was determined by examining the fluorescence responses of P1 and P2–GO toward pc-DNA T1 and sm-DNA T2. Figure 5A displays the fluorescence emission spectra of P1 in the presence of fivefold T1 and T2. The fluorescence intensity enhancement value by T2 (curve b) was 78% of

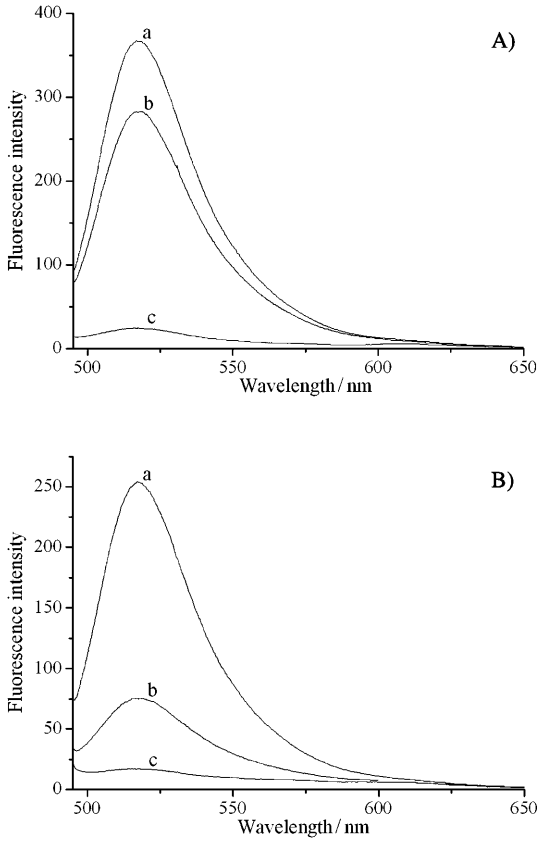


Figure 5. Changes in the fluorescence emissions of P1 and P2–GO. A) Fluorescence emission spectra of P1 (curve c) with a fivefold excess of T1 (curve a) or fivefold excess of T2 (curve b). B) Fluorescence emission spectra of P2–GO (curve c) with a fivefold excess of T1 (curve a) or fivefold excess of T2 (curve b).

that by T1 (curve a). Meanwhile, as shown in Figure 5B, the fluorescence intensity enhancement value of P2–GO by the T2 (curve b) was 30% of that by T1 (curve a). These results reveal that the single-base mismatch selectivity of P2–GO is significantly greater than that of P1.

In order to demonstrate the single-base-mismatch selectivity of the GO-quenched MB more clearly, we introduced a selectivity coefficient, α, defined as $\alpha=(S/B)_{i\text{-}j}/(S/B)_{i\text{-}j'}$, where $(S/B)_{i\text{-}j}$ is the S/B value for the MB, i, in the presence of DNA target, j, and $(S/B)_{i\text{-}j'}$ is that for the same MB in the presence of target, j'.[13] Here we used the selectivity of each MB for T1 as the standard ($\alpha=1$). Therefore, we can calculate the selectivity coefficient of P1 and P2–GO. The selectivity coefficient of P1 for T2 over T1 was $\alpha=0.732$, while the selectivity coefficient of P2–GO for T2 over T1 was $\alpha=0.246$. We also calculated the selectivity coefficient of P3 and P4–GO. The selectivity coefficient of P3 for T2 over T1 was $\alpha=0.783$, while the selectivity coefficient of P4–GO for T2 over T1 was $\alpha=0.267$. The results show that the use of

GO as the "nanoquencher" can greatly improve the single-base-mismatch selectivity of an MB. The enhanced selectivity might be due to the conformational restriction of the HP-structured oligonucleotides on the graphene surface. It must be mentioned that the use of SWNTs as "nanoquencher" only slightly improves the single-base-mismatch selectivity.[13]

The efficiency of GO was also compared with that of SWNTs. With their exceptional quenching capability, SWNTs have been successfully used to construct MBs. When used as the quencher in an MB, the average quenching efficiency of SWNTs to FAM has been shown to be as high as 98%. GO showed a higher quenching efficiency (99.1%) than SWNTs. In addition, the use of GO with MBs can greatly improve their DNA detection sensitivity and single-base-mismatch selectivity. Furthermore, compared to the SWNTs, GO is easier to prepare and manipulate.

Kinetic behavior of GO-quenched MBs: The kinetic properties of GO binding and subsequent DNA hybridization of the fluorescent oligonucleotide are fundamentally different from those of conventional MBs. Figure 6, curve a shows the

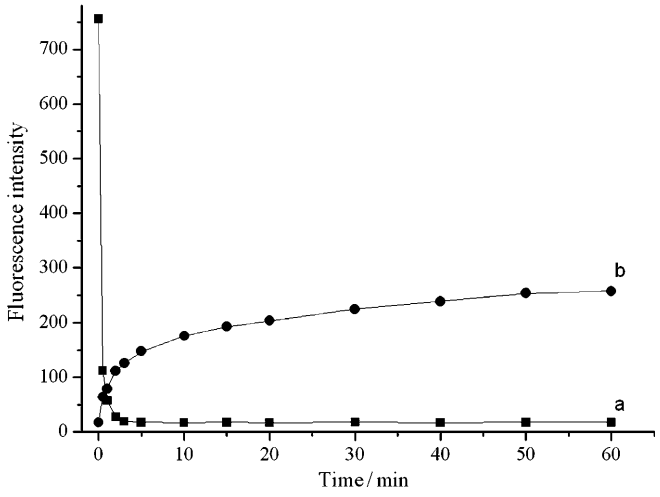


Figure 6. Fluorescence quenching of P2 (50 nM) by GO as a function of time (curve a) and fluorescence restoration of P2–GO by T1 (250 nM) as a function of time (curve b).

fluorescence quenching of P2 in the presence of GO as a function of incubation time. Single-stranded DNA adsorption on the surface of GO is very fast at room temperature. It reaches equilibrium in 5 min. However, the formation and release of the double-stranded DNA from GO is relatively slow and needs almost 1 h (Figure 6, curve b). It must be mentioned that hybridization of GO-quenched MB and target DNA is slower than that of conventional MBs and target DNA (conventional MB P1 and fivefold T1 hybridization reaches equilibrium in 30 min). This difference could be because the HP-structured oligonucleotide is strongly adsorbed on GO surface, thus decreasing the probability and tendency of hybridizing with complementary DNA.

Conclusion

In summary, we report a new highly sensitive, selective and economical method that uses molecular beacons with graphene oxide as the "nanoquencher". The key components of the novel graphene oxide-quenched molecular beacon are the HP-structured fluorescent oligonucleotide and graphene oxide sheet, which functions as both "nanoscaffold" for the oligonucleotide and "nanoquencher" for the fluorophore. The proposed graphene oxide-quenched MB has three distinguished features. 1) The graphene oxide-quenched MB needs only one labeled fluorophore. This design provides a more convenient one-step synthesis/purification protocol compared to conventional molecular beacons. 2) Graphene oxide shows exceptionally high fluorescence quenching efficiency. The graphene oxide-quenched MB method has a higher sensitivity than conventional molecular beacon methods. 3) Using graphene oxide can greatly improve the single-base-mismatch selectivity of a molecular beacon because of the conformational constraint of the HP-structured oligonucleotide on the graphene oxide surface. Due to the cellular delivery ability of graphene oxide, graphene oxide-quenched MBs could be applied to challenging applications involving cellular and physiological problems. Currently, intensive research on the use of graphene oxide-quenched molecular beacons for imaging of mRNA in live cells is being conducted in our laboratory, and will be communicated in due course.

Experimental Section

General: Oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co. (China). Fluorescence measurements were performed on a Hitachi F-4600 fluorometer (Hitachi Co. Ltd., Japan). AFM images were recorded by using a Nanoscope IIIa multimode atomic force microscope (Veeco Instruments, USA) in tapping mode to simultaneously collect height and phase data.

Synthesis of graphene oxide (GO): GO was synthesized from natural graphite powder by a modified Hummers method.[21] Briefly, graphite powder (2 g) was ground with NaCl to reduce the particle size. After removing the salt, the graphite was added to concentrated H_2SO_4 (80 mL) and stirred for 2 h. Afterwards, $KMnO_4$ (10 g) was added gradually while the solution was stirred and the temperature of the mixture was kept to less than 20 °C. Successively, the mixture was stirred at 35 °C for 2 h. The temperature was maintained below 50 °C and distilled water (180 mL) was added; then the mixture was stirred at room temperature for 3 h. The reaction was ended by a final addition of distilled water (450 mL) and H_2O_2 (30%, 20 mL) solution. Finally, the mixture was repeatedly washed with 1:10 HCl aqueous solution, and then with distilled water. Exfoliation was carried out by sonicating the GO (2 mg mL^{-1}) dispersion under ambient conditions for 4 h.

Fluorescence quenching and hybridization assays: The working solution containing the fluorescent oligonucleotide was obtained by dilution of the stock solution to a concentration of 50 nM by using Tris-HCl buffer (20 mM, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$). An aliquot of the GO suspension (about 0.04 mg mL^{-1}) was added to the fluorescent oligonucleotide (P2 or P4) working solution and incubated for 5 min. A fivefold molar excess of complementary target T1 was then added to the GO–fluorescent oligonucleotide mixture and allowed to hybridize for 2 h at room temperature. Afterwards, the fluorescence of the mixture was detected. The control solution without target was obtained

by addition of the same volume of water to the GO–fluorescent oligonucleotide mixture solution. In order to compare the molecular recognition ability of the GO-quencher beacon with that of the conventional MBs, similar titrations were carried out with the conventional MBs (P1 and P3), which had dabcyl as the quencher.

Acknowledgements

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[1] A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.* **2008**, *108*, 109–139.
[2] A. A. Martí, S. Jockusch, N. Stevens, J. Y. Ju, N. J. Turro, *Acc. Chem. Res.* **2007**, *40*, 402–409.
[3] C. Batchelor-McAuleya, G. G. Wildgoosea, R. G. Compton, *Biosens. Bioelectron.* **2009**, *24*, 3183–3190.
[4] S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303–308.
[5] K. M. Wang, Z. W. Tang, C. Y. J. Yang, Y. M. Kim, X. H. Fang, W. Li, Y. R. Wu, C. D. Medley, Z. H. Cao, J. Li, P. Colon, H. Lin, W. H. Tan, *Angew. Chem.* **2009**, *121*, 870–885; *Angew. Chem. Int. Ed.* **2009**, *48*, 856–870.
[6] G. Bonnet, S. Tyagi, A. Libchaber, F. R. Kramer, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6171–6176.
[7] D. L. Sokol, X. Zhang, P. Lu, A. M. Gewirtz, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11538–11543.
[8] X. H. Fang, Y. M. Mi, J. W. J. Li, T. Beck, S. Schuster, W. H. Tan, *Cell Biochem. Biophys.* **2002**, *37*, 071–081.
[9] D. P. Bratu, B. J. Cha, M. M. Mhlanga, F. R. Kramer, S. Tyagi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13308–13313.
[10] M. M. Mhlanga, D. Y. Vargas, C. W. Fung, F. R. Kramer, S. Tyagi, *Nucleic Acids Res.* **2005**, *33*, 1902–1912.
[11] P. Santangelo, N. Nitin, L. Laconte, A. Woolums, G. Bao, *J. Virol.* **2006**, *80*, 682–688.
[12] B. Dubertret, M. Calame, A. J. Libchaber, *Nat. Biotechnol.* **2001**, *19*, 365–370.
[13] R. H. Yang, J. Y. Jin, Y. Chen, N. Shao, H. Z. Kang, Z. Y. Xiao, Z. W. Tang, Y. R. Wu, Z. Zhu, W. H. Tan, *J. Am. Chem. Soc.* **2008**, *130*, 8351–8358.
[14] K. S. Novoselov, A. K. Geim, S. V. Morozov, D. Jiang, Y. Zhang, S. V. Dubonos, I. V. Grigorieva, A. A. Firsov, *Science* **2004**, *306*, 666–669.
[15] A. K. Geim, K. S. Novoselov, *Nat. Mater.* **2007**, *6*, 183–191.
[16] G. Eda, M. Chhowalla, *Nano Lett.* **2009**, *9*, 814–818.
[17] E. J. Yoo, J. Kim, E. Hosono, H. Zhou, T. Kudo, I. Honma, *Nano Lett.* **2008**, *8*, 2277–2282.
[18] N. Mohanty, V. Berry, *Nano Lett.* **2008**, *8*, 4469–4476.
[19] C. S. Shan, H. F. Yang, J. F. Song, D. X. Han, A. Ivaska, L. Niu, *Anal. Chem.* **2009**, *81*, 2378–2382.
[20] C. H. Lu, H. H. Yang, C. L. Zhu, X. Chen, G. N. Chen, *Angew. Chem.* **2009**, *121*, 4879–4881; *Angew. Chem. Int. Ed.* **2009**, *48*, 4785–4787.
[21] W. S. Hummers, R. E. Offeman, *J. Am. Chem. Soc.* **1958**, *80*, 1339–1339.
[22] X. B. Fan, W. C. Peng, Y. Li, X. Y. Li, S. L. Wang, G. L. Zhang, F. B. Zhang, *Adv. Mater.* **2008**, *20*, 4490–4493.
[23] S. A. E. Marras, F. R. Kramer, S. Tyagi, *Nucleic Acids Res.* **2002**, *30*, 122e.
[24] D. C. Altieri, *Oncogene* **2003**, *22*, 8581–8589.
[25] Z. Liu, J. T. Robinson, X. M. Sun, H. J. Dai, *J. Am. Chem. Soc.* **2008**, *130*, 10876–10877.
[26] M. Zheng, A. Jagota, E. D. Semke, B. A. Diner, R. S. McLean, S. R. Lustig, R. E. Richardson, N. G. Tassi, *Nat. Mater.* **2003**, *2*, 338–342.
[27] N. Varghese, U. Mogera, A. Govindaraj, A. Das, P. K. Maiti, A. K. Sood, C. N. R. Rao, *ChemPhysChem* **2009**, *10*, 206–210.

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