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A graphene-based platform for fluorescent detection of SNPs†

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A novel fluorescent single nucleotide polymorphism (SNP) assay was developed by using Graphene Oxide (GO), which provides a fast, sensitive and simple method for SNP detection. The strategy was based on the single base extension reaction and different absorption capacity of fluorescein labeled dGTP (dGTP-Fl) and double-stranded DNA (dsDNA) to GO. dGTP-Fl is incorporated into the probe by extension reaction for the mutant target but not for the wild target, which leads to recovered fluorescence for the mutant target because of weak interaction between dsDNA and GO and weak fluorescence for the wild target because of the quenched fluorescence of dGTP-Fl by GO. The method shows a linear range for the mutant-type target from 3 nM to 50 nM and 3 nM is the detection limit. It was noted that as low as 10% mutant-type target could be detected in the presence of the wild-type target, in which the concentration is 9 times higher than that of the mutant-type target.

Introduction

Single nucleotide polymorphisms (SNPs) are the polymorphisms which are induced by variation of a single nucleotide in the genome and represent a natural genetic variability at high density in the human genome.[1] Genotyping of SNPs will help us gain a deep understanding of complex diseases and identify disease-causing genes.[1,2] The determination of SNPs is in high demand not only because of the important part it plays in preventing diseases, selecting medicines, developing new medicines and vaccinating diseases,[3,4] but also in studying the genome structure and function.[1,2,5,6] Various methods have been reported to detect SNP,[1,6–8] such as single-strand conformation polymorphism analysis, heteroduplex analysis, allele specific oligonucleotide hybridization, enzyme mismatch cleavage, oligonucleotide ligation, and invader assays. However, most of these methods are based on gel electrophoresis or heterogeneous assay formats, which are laborious and time-consuming due to the multiple separation or washing steps. Fluorescence-based methods in homogeneous solution have been widely used but the requirement for double labeled DNA probes leads to high cost and complex procedures. Recently, nanomaterials-based sensing strategies, including silver nanocluster probe,[9] copper nanoclusters,[10] and conjugated polymers,[11–13] have been developed to detect SNP with good sensitivity and selectivity. However, further intense study is needed to detect SNP with improved sensitivity and selectivity due to the importance of SNP monitoring. Here, we proposed to detect SNP by using graphene oxide (GO) to satisfy these requirements.

Graphene is a multifunctional nanomaterial comprising one-atom-thick planar sheets of sp^2-bonded carbon atoms,[14,15] which has extraordinary electronic, thermal and mechanical properties.[16–21] GO, the water-soluble derivative of graphene, has been widely applied in nanoelectronics,[22] sensors[23–26] and nanocomposites.[27–29] As a new material in biological applications, GO represents excellent aqueous solubility and biocompatibility in virtue of the exposed carboxyl and hydroxyl groups on the surface of GO sheets, and it has great potential for use in biosensors, bioanalysis and biomedicine. GO could be used for distinguishing single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) due to the preferential binding of GO to ssDNA over dsDNA, which was considered to be related to the hydrophobic and π–π stacking interactions between GO and nucleobases.[30] GO is also recognized as an effective quencher for a variety of fluorophores and greatly decreases the background noise due to high efficient long-range energy transfer from the dye to GO.[26,31,32] In the presence of GO, the fluorescein labeled ssDNA was absorbed to the GO sheet and the fluorescence (FL) was intensively quenched. However, the weak interaction of dsDNA and GO kept the fluorescein apart from GO and a high signal was recorded. Based on this characteristic, many DNA sensors have been established.[26,33]

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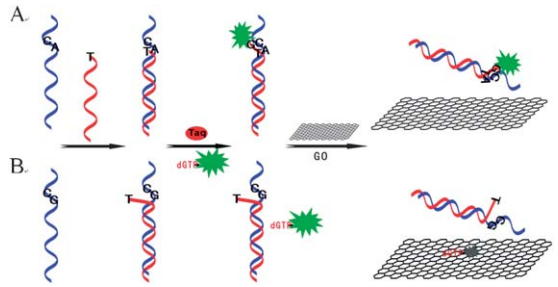

Scheme 1 Schematic description of the GO–SNP sensing mechanism. The T at the 3′-terminal of the probe sequence is not complementary to the G in the wild target sequence and dGTP-Fl cannot be incorporated into the probe, leading to quenched FL by GO. While T is complementary to A in the mutant-type target and dGTP-Fl is incorporated into the probe by an extension reaction, leading to recovered FL due to the weaker interaction of dsDNA with GO.

Here, we developed a novel strategy through introduction of GO into a single base extension (SBE) reaction which is widely used in SNP discrimination.[7,34–36] Here, the SBE reaction used a primer with the 3′-terminal base complementary to the mutant target sequence but not to the wild target sequence.[7] Upon addition of a polymerase and the fluorescein labeled dGTP (dGTP-Fl), only those primers complementary to the mutant target are extended and dGTP-Fl is incorporated into the probe by an extension reaction for the mutant target, leading to fluorescein labeled dsDNA and recovered FL. For the wild target sequence, dGTP-Fl cannot be incorporated into the probe during the extension reaction and is absorbed on the GO sheet, leading to quenching of FL. Furthermore, fluorescent dye and nucleobases possess high affinity with GO,[26,31,37] thus dGTP-Fl is considered to be quenched by GO with high efficiency due to its small molecular size and high affinity. This design always leads to a low FL background and a high signal to noise ratio, as a consequence, a high sensitivity and good selectivity will be achieved in SNP assay with this GO-based SBE method. As illustrated in Scheme 1, the target DNA fragment is part of p53exon8 containing a polymorphic site (Arg282Trp), at which the nucleotide A was designed in the mutant target sequence instead of G in the wild target sequence. The 3′-terminal base of the probe sequence is T which is complementary to A in the mutant-type target sequence but not complementary to G in the wild target sequence. dGTP-Fl was used for probe extension reactions in the presence of Taq DNA polymerase. dGTP-Fl is incorporated into the probe by extension reaction for the mutant target but not for the wild target, which leads to recovered FL for the mutant target because of weak interaction of dsDNA and GO and weak FL for the wild target because of quenched FL of dGTP-Fl by GO.

Experimental

Materials and measurements

DNA oligonucleotides were synthesized and purified by TAKARA Biotechnology Corporation (Dalian, China). The sequences of these oligonucleotides are shown in Table 1. dGTP-Fl was obtained from Perkin Elmer. Graphite powder was purchased from China National Pharmaceutical Group Corporation (Shanghai, China). GO was synthesized from the natural graphite powder by using the modified Hummer's method[38] and characterized by using a tapping-mode atomic force microscope (AFM, Fig. S1†). Other chemicals were of analytical grade from China National Pharmaceutical Group Corporation. Water was purified using a Millipore filtration system. Centrifugation was carried out using a Centrifuge Himac-CF 16RX (Hitachi, Japan). All the base extension reactions were carried out in Takara Taq reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3) in a Bioer Little Genius TC-25/H Thermal Cycler (Hangzhou, China). The FL spectra were measured with a Hitachi F-4500 spectrofluorimeter equipped with a xenon lamp excitation source in 25 mM HEPES buffer (λ_{ex} = 494 nm, λ_{em} = 527 nm).

Table 1 The DNA sequences used in our work

DNA	5′–3′
Probe	TGCCTGTCCTGGGAGAGACT
Mutant target	CCTCTGTGCGCCAGTCTCTCCCAGGACAGGCA
Wild target	CCTCTGTGCGCCGGTCTCTCCCAGGACAGGCA

SBE reaction

For each different allele-specific primer, 24 μL Reaction Master Mixture (MM) was prepared, containing 3 μL 10× PCR buffer, 3 μL dGTP-Fl (40 μM), 6 μL probe (10 μM), 4 Unit Taq DNA polymerase (0.8 μL) and 11.2 μL MilliQ-H_2O. 12 μL of each MM was pipetted into a 200 μL PCR microtube kept on ice, and then 3 μL of DNA target sample (10 μM) was individually added. For the single-base primer extension reaction, the temperature profile consisted of 1 cycle of 4 min at 94 °C, 59 cycles of 30 s at 94 °C and 30 s at 55 °C, 1 cycle of 10 min at 55 °C. After the reaction was completed, the product was kept at 4 °C. The SBE products were further confirmed by native polyacrylamide gel-electrophoresis (PAGE) analysis and the FL was observed through fluorescein. For FL measurement, 15 μL SBE reaction solution was diluted with HEPES buffer to 700 μL, and 45 μL of GO (0.47 g L^{-1}) was added and additional HEPES were added to makeup the detection solution to 800 μL. The FL emission spectra were recorded with a spectrofluorimeter equipped with a xenon lamp excitation source (λ_{ex} = 494 nm).

Optimization of GO usage and GO quenching kinetics

The usage of GO was optimized to reach the best performance of this FL sensor. 15 μL extension products were diluted with HEPES to 700 μL, then different volumes of GO (0.47 g L^{-1}) were used and additional HEPES were added to makeup the detection solution to 800 μL. The final concentrations of GO in the cuvette were 0, 2.94, 5.88, 8.81, 11.75, 14.69, 17.63, 20.56, 23.5, 26.4, 29.4, 32.3 mg L^{-1}, respectively.

The kinetics experiment was studied for mutant target-containing and wild target-containing SBE products in the presence of 23.5 mg L^{-1} GO by using the time scanning model on the spectrofluorimeter.

The kinetics experiment in the presence of GO was carried out after adding GO for 15 s, and this time gap was chosen to mix the solution and to carry out other necessary procedures for detection.

DNA detection sensitivity and allele frequency analysis

A series of concentrations of mutant target (0, 3, 5, 10, 25, 50 nM) containing SBE products were mixed with 26.4 mg L^{-1} GO to obtain the linear range and detection limit. To determine the allele frequency, samples with different content of wild target and mutant target were tested with GO in the spectrofluorimeter. In the samples, the contents of mutant target were 0%, 10%, 20%, 50%, 70%, 90% and 100%, respectively, and the total concentration of mutant and wild-type target was 5×10^{-8} M. All the spectra except for the kinetics experiment were recorded after 65 s upon addition of GO.

Results and discussion

The sensing mechanism for SNP

In Scheme 1, the probe is mixed with the target sequences (mutant target and wild target) and hybridization is completed in the thermal cycler. For the probe sequence to be fully complementary to the mutant target sequence, the base extension reactions can be performed in the presence of Taq DNA polymerase (Scheme 1A). In Scheme 1B, the T at the 3′-terminal of the probe sequence is not complementary to the G in the wild target sequence and dGTP-Fl cannot be incorporated into the probe. The SBE products were characterized by PAGE (Fig. S2†). The FL spectra were recorded and no significant difference was found for both wild and mutant targets in the absence of GO (data not shown). However, upon adding GO, for the extension products of mutant target, dsDNA-Fl cannot be absorbed onto the GO sheet efficiently and strong FL intensity is obtained (Scheme 1A). For the wild target, dGTP-Fl is absorbed onto the GO sheet and weaker FL is recorded due to the efficient quenching of the GO sheet by the dye (Scheme 1B). As indicated in Fig. 1, the FL intensity presents significant difference between the mutant target and wild target when the concentration of GO is 26.4 mg L^{-1}. The FL intensity of the mutant target is nearly 4.2 times higher than that of the wild target, indicating the validity of the method for SNP detection by combining the SBE reaction and GO. The background FL of the mixture of GO and HEPES buffer was deducted.

Optimization of the concentration of GO

The detection of SNP in our work was evaluated by FL measurements of fluorescein in the presence of GO. According to previous work,[26,30] the FL quenching depended on the ratio of GO and fluorescein labeled DNA. Based on it, different concentrations of GO were used to optimize the detection efficiency of SNP in the present experiment, and the optimized conditions were investigated in the presence of 0, 2.94, 5.88, 8.81, 11.75, 14.69, 17.63, 20.56, 23.5, 26.4, 29.4, 32.3 mg L^{-1} of GO, respectively. As shown in Fig. 2a and Fig. 2b, the FL intensity decreases both for the mutant target and for the wild target when the concentration of GO is increased. Fluorescence decrease of the former (F_m) is much slower than the latter (F_w), and the F_m/F_w was calculated to reach the best performance of this sensor. The highest ratio was 4.2, at which the concentration of GO is 26.4 mg L^{-1}.

Kinetics study of GO absorption to DNA for SNP detection

Time-scan spectrometry was carried out after the addition of GO to mutant or wild target products in the presence of PCR buffer and Taq DNA polymerase. In Fig. 3b, it was demonstrated that in the wild target products, the FL intensity decreased sharply from 518 to 350 within 30 s after the addition of GO. However, in mutant target products, the FL intensity decreased slowly from 1100 to 900 within 5 min after the addition of GO, and then it leveled off (Fig. 3a). The highest F_m/F_w was obtained after adding GO for 50 s (Fig. 3c). In fact, we could discriminate the mutant target from the wild one at any timepoints after adding GO since more than 2 of the F_m/F_w was sufficient for detection.

Sensitivity of mutant-type target detection

The GO based SNP assay was evaluated by applying it in the determination of a series of mutant-type target concentrations (from 0 to 50 nM) under the optimized conditions. As shown in Fig. 4A, the FL emission intensity increased when the concentration of the mutant-type target increased. The method

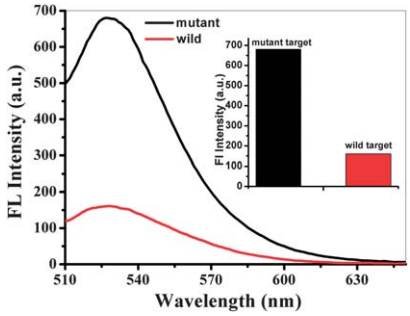

Fig. 1 FL spectra of the mutant target and wild target when the concentration of GO is 26.4 mg L^{-1}. The concentration of mutant or wild-type target is 5×10^{-8} M, [probe] = 5×10^{-8} M, [dGTP-Fl] = 2×10^{-7} M.

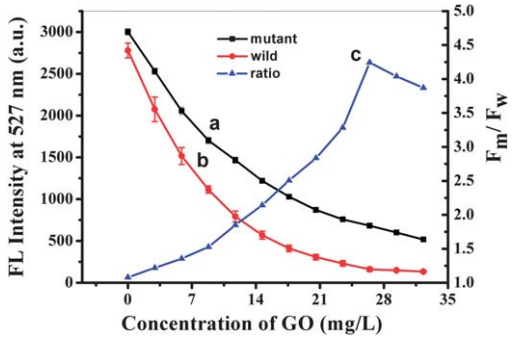

Fig. 2 FL intensity of (a) mutant target, (b) wild target, and (c) F_m/F_w ratio in the presence of different concentrations of GO (0, 2.94, 5.88, 8.81, 11.75, 14.69, 17.63, 20.56, 23.5, 26.4, 29.4, 32.3 mg L^{-1}).

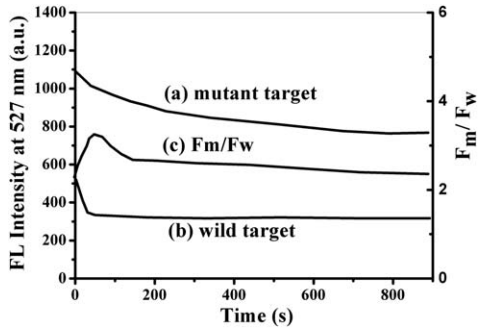

Fig. 3 The kinetics behavior of (a) mutant target-containing and (b) wild target-containing SBE products on the surface of GO. (c) The calculated F_m/F_w. The concentration of mutant or wild-type target is 5×10^{-8} M, [probe] = 5×10^{-8} M, [dGTP-Fl] = 2×10^{-7} M, [GO] = 23.5 mg L^{-1}.

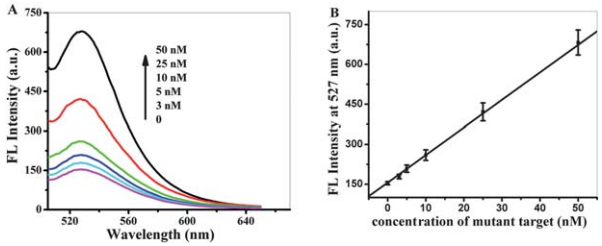

Fig. 4 Fluorescence spectra (A) and FL intensity at 527 nm (B) of solutions containing GO, probe and dGTP-Fl with different concentrations of mutant-type target (0, 3, 5, 10, 25, 50 nM). [Probe] = 5×10^{-8} M, [dGTP-Fl] = 2×10^{-7} M, [GO] = 26.4 mg L^{-1}. The same experiment was repeated three times to obtain the error bar.

exhibited a linear response toward concentration of mutant-type target from 3 nM to 50 nM (Fig. 4 (B)). The calibration equation was $y = 156.5036 + 10.3412x$ (y: FL intensity, x: the concentration of mutant-type target (nM), $R = 0.9962$). As low as 3 nM mutant-type target could be detected, which is comparable or better than most SNP assays (Table S1†). The results indicate that the present method can be successfully applied in the detection of SNP with high sensitivity.

Allele frequency estimation

For the association study of SNPs and diseases, it is required to quantify the allele frequency. In our work, mixed samples with different ratios of mutant target were adopted for FL assay. Samples at various allele frequencies with the mutant target and wild target DNA fragments mixed at various ratios of 0, 10%, 20%, 50%, 70%, 90%, 100% were tested. The total concentration of mutant and wild target was 5×10^{-8} M. The FL emission spectra and the statistic curve of the relationship between the $FL_{527\ nm}$ and the percentage of mutant target in the test sample are shown in Fig. 5. The increase of the ratio of mutant target in the test sample led to a gradual increase of the FL intensity at 527 nm, and as low as 10% mutant target was detected. It was illustrated that the mutant target could be detected even in the presence of wild target, the concentration of which was 9 times higher than that of the mutant target.

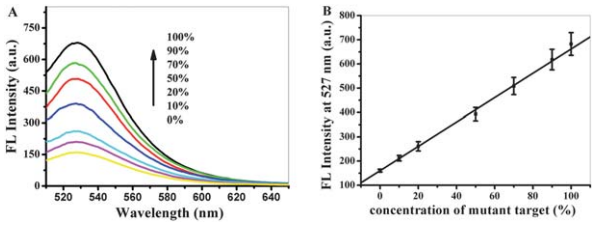

Fig. 5 (A) Emission spectra of the extension production at various allele frequencies. (B) FL intensity at 527 nm as a function of allele frequencies. The total concentration of mutant and wild-type target is 5×10^{-8} M, [probe] = 5×10^{-8} M, [dGTP-Fl] = 2×10^{-7} M, [GO] = 26.4 mg L^{-1}. The excitation wavelength is 494 nm. The same experiment was repeated three times to obtain the error bar.

Conclusions

In our work, SNP detection with good sensitivity and selectivity was achieved based on the SBE reaction and different absorption capacity of dGTP-Fl and dsDNA to GO. Several advantages were presented in this method. (1) The SBE method possesses good selectivity and high sensitivity, and here dGTP-Fl is used to incorporate into the probe by an extension reaction for the mutant target. The FL arising from the mutant target group was the long sequence of the Fl-tagged PCR product, while that from the wild group was the free dGTP-Fl, which was easily differentiated by GO. (2) Small size and high affinity contribute to the efficient FL quenching of GO to dGTP-Fl, as a consequence, low FL noise and high signal to noise ratio, together with high sensitivity and selectivity are achieved. Under the optimized conditions, the FL intensity of the mutant target sample is 4.2 times higher than that of the wild target sample. The method presented here shows a linear range for the mutant-type target of 3 nM to 50 nM and the detection limit is 3 nM, which shows that the method demonstrates high sensitivity. In the target mixture analysis, as low as 10% mutant target can be detected. This enzyme based detection combines the advantages of SBE reaction and efficient quenching of GO to dGTP-Fl, which is highly sensitive and cost-effective. It shows great potential in SNP detection of disease related diagnosis.

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