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RNA detection based on cyclic amplification of graphene oxide fluorescence

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Abstract. – OBJECTIVE: Graphene oxide (GO) was prepared and used to adsorb single-stranded DNA. Based on fluorescence quenching ability of GO, cyclic signal amplification was performed using polymerase. Thus, two RNA sequences were detected by fluorescence assay.

METHODS: Klenow fragment was designed for the amplification of fluorescence signals and RNA was detected by fluorescence assay. After amplification with Klenow fragment, the lowest limit of detection was 1.0×10^{-11} M, and the response of fluorescent intensity was linear within the concentration range of 1.0×10^{-11} M -1.0×10^{-9} M. By the modification of DNA terminals with different fluorescent groups, two different RNA were detected.

RESULTS: Different fluorescent dyes were used to modify the terminals of DNA, and two RNA sequences were detected based on fluorescence. There was no need for product separation and purification before detection.

CONCLUSIONS: Detection of RNA based on cyclic amplification of GO fluorescence is fast and simple. It can be used for the analysis of specific RNA sequences in cancer cells.

Key Words:
Graphene oxide, RNA, Signal amplification, Fluorescence assay.

Introduction

Malignant tumors pose a great threat to human health, and the incidence is rising at a fast rate. There are about over 10 million newly diagnosed cases of malignancies throughout the world. According to the statistics by WHO in 2008, the deaths caused by cancers account for 13% of all deaths globally. In the US and Singapore, cancer-related deaths account for 1/4-1/3 among all deaths of the country. Cancer is only secondary to cardiovascular disease as the leading cause of death in these countries. Although the survival rate of cancer patients has improved markedly in recent years, cancer mortality remains basically unchanged. The improvement of survival rate is mainly attributed to an early diagnosis of cancers and improved treatment techniques. In western countries, acute leukemia, malignant lymphoma and multiple myelomas are among the top ten cancers regarding incidence. The incidence of these cancers is also rising in China. There are about 25 thousand newly diagnosed cases of lymphoma in China. The cases of non-Hodgkin lymphoma now account for 3%-5% with the fastest increase of incidence among all cancers. The common diagnostic techniques for cancers include pathological examination and radiological examination. However, biopsies usually cause considerable trauma and pain to the patients. For bone tumors, a small specimen size will affect the pathological result, and it takes longer to make a final diagnosis. Radiological examination has difficulty in identifying tumor lesions less than 2 mm and the diagnosis must be confirmed by other techniques. Nanotechnology is an emerging technique in tumor diagnosis and treatment. The application of nanotechnology in biomedicine and pharmaceuticals has revolutionized the field of biomedicine. Graphene oxide (GO) is a novel 2D nanomaterial belonging to the carbon family. GO usually exists in sheets as the product of exfoliation of the graphite oxide. GO is stable in aqueous solutions, and after dehydration, GO is reduced to multi-layer graphite oxide. Compared with graphene, GO contains the oxygen-containing groups such as carboxyl groups and hydroxyl groups. GO is very easily dissolved in water as opposed to the low solubility of graphene in water. The common methods for preparing GO include Brodie method, Staudenmaier method and Hummers method and

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their corresponding modifications. Hummers method[1,2] involves the use of concentrated sulfuric acid and sodium nitrate as strong protonic acid and potassium permanganate as a strong oxidant. This method is environmental-friendly, which has higher safety and higher performance of the products compared with the other two procedures. In this study, we prepared GO by Hummers method and preserved GO in vacuum at low temperature and low humidity. Ultrasonic exfoliation was performed before use to remove the unoxidized graphite and to obtain single-layer or multilayer GO solution. The non-covalent π-π stacking interaction between the hexagonal crystal system of GO and the circular structure of nucleic acid base results in adsorption ability on single-stranded DNA. Since the bases are not exposed on double-stranded DNA, GO cannot adsorb double-stranded DNA. This feature is usually used to separate the single-stranded DNA. The experiment shows that GO is more likely to adsorb long oligonucleotides containing a larger number of deoxynucleotide as compared with short oligonucleotides. Many researches have been carried out by utilizing the adsorption ability of GO. Tang et al[3] incubated two DNA sequences with GO separately before mixing them together, and then the third sequence was added for hybridization with the first two DNA sequences. Finally, grain size analysis was performed.

GO can easily adsorb single-stranded DNA, thus modifying the terminals of single-stranded DNA with a fluorescent dye. As a result, the fluorescent dye on the surface of GO will jump to the excited state from the ground state after absorbing the excitation light. The photons released during the jump from the lowest level of excited state to the ground state will be absorbed by GO. Thus, the fluorescence is quenched. GO is an excellent fluorescence quencher for various fluorescent dyes because of fluorescence resonance energy transfer (FRET) over extensive nanoscale. Many experiments have been conducted based on fluorescence quenching ability of GO. Li et al[4] adsorbed the FAM-labeled single-stranded DNA using GO, thus transferring the fluorescent dye from the solution to the surface of GO. As the fluorescence was quenched by FRET, bleomycin was added into the solution so as to recognize and cleave the DNA sequences. Consequently, the DNA sequence either became shorter or was hydrolyzed into deoxynucleotide, and GO could no longer adsorb the oligonucleotide. The FAM-labeled groups were released into the solution. One molecule of bleomycin corresponded to several oligonucleotide molecules, and a large number of fluorescent materials accumulated into solution. Using this method, at least 5 nM of bleomycin can be detected.

We proposed the method of RNA detection based on cyclic amplification of GO fluorescence. Two RNA sequences were detected by measuring the fluorescence signals.

Experimental Equipments and reagents

Equipments

Electrochemical workstation (CHI660E, CH Instruments, Bee Cave, TX, USA), transmission electron microscope (JEM-2100, Hitachi, Tokyo, Japan), UV-Vis Spectrophotometer (Cary 60, Agilent, Santa Clara, CA, USA), fluorescence spectrophotometer (F-4600, Hitachi, Tokyo, Japan), pH meter (PHS-3C, Shanghai Precision Instrument Co., Ltd. Shanghai, China), numerical water bath temperature controller (Beijing Chang'an Scientific Instrument Factory, Beijing, China), ultra-pure water system (UPT-II-60L, Chengdu Ultrapure Technology Co., Ltd. Chengdu, Sichuan, China), electronic balance (FA2004B, Shanghai Precision Instrument Co., Ltd. Shanghai, China), magnetic stirrer (CL-2, Gongyi City Yuhua Instrument Co., Ltd. Gongyi, Henan, China), adjustable pipette (Biohit), digital control ultrasonic cleaner (KH-200KDE, Kunshan Hechuang Ultrasonic Equipment Co., Ltd. Kunshan, Jiangsu, China), vacuum oven (DZF-6030A, Shanghai Yiheng Scientific Instruments Co., Ltd. Shanghai, China), dialysis bag MD34 (7000D, Union Carbide, Houston, TX, China), gas bath thermostatic oscillator (CHA-S, Jiangsu Jintan Medical Instrument Factory, Jintan, Jiangsu, China).

Reagents

DNA sequence (Sangon Biotech Co., Ltd., Shanghai, China) (Table I), RNA sequence (Suzhou GenePharma, Suzhou, Jiangsu, China) (Table I), Klenow fragment (Sangon Biotech (Shanghai) Co., Ltd. Shanghai, China), graphite powder (Sinopharm Chemical Reagent Co., Ltd. Shanghai, China), 30% hydrogen peroxide solution (Laiyang Economic and Technological Development Zone Fine Chemical Plant. Yantai, Shandong, China), carboxyl-labeled magnetic microsphere (MB-COOH, 10 g/L, BaseLine Chromtech Research Center, Tianjin, China). All solutions were prepared by the standard method. All

Table I. DNA/RNA sequences.

DNA	DNA sequence (5'-3')
DNA1	5'-ACT ATA CAA CCT ACT ACC TCA TTT TT-NH2-3'
DNA2	5'-AAA CTC CAT TGT CAC ACT CCA TTT TT-NH2-3'
DNA3	5'-AGG TTG TAT AGT GTG ACC-3'
DNA4	5'-ACA ATG GAG TTT GTG ACC-3'
DNA5	5'-GGT CAC ACT ATA CAA CCT GCG ACG GC-FAM-3'
DNA6	5'-GGT CAC AAA CTC CAT TGT GCG ACG GC-Cy5-3'
DNA7	5'-Pi-GCC GTC GC-3'
RNA1	5'-UGA GGU AGU AGG UUG UAU AGU U-3'
RNA2	5'-UGG AGU GUG ACA AUG GAG UUU G-3'

solutions used for detecting RNA were prepared with 0.1% Diethyl pyrocarbonate (DEPC)-treated water after high-pressure sterilization. Other reagents were analytically pure.

Methods

Preparation of GO

GO was prepared using Hummers method with the greater amount of oxidant and sulfuric acid and longer reaction time. Into the round-bottom flask 0.5 g carbon powder, 0.3 g sodium nitrate and 25 ml concentrated sulfuric acid were added successively. The flask was placed in ice water bath and stirred at low speed. During stirring, 3 g of potassium permanganate was added in several times and the temperature of the solution was maintained below 10°C using a thermometer. The solution was further stirred for 1h in ice water bath, and the flask was transferred to 35°C water bath, followed by fast stirring for 2h. The sample became thicker during stirring. The flask was then transferred to boiling water and 23 ml water was added slowly using a constant pressure funnel. The temperature of the solution was maintained below 98°C during stirring until the sample became extremely thick. Fast stirring was performed in boiling water for 15 min, and 3% hydrogen peroxide solution was added until the solution became bright yellow. The solution was passed through 0.2 μm filter and washed with hot diluted hydrochloric acid solution three times to obtain a dark brown filter cake. The filter cake was dispersed in 30 ml water and added with a small amount of diluted hydrochloric acid and centrifuged. The precipitate collected was dissolved in 30 ml water and passed through 7000 Dalton dialysis bag. The water was replaced twice daily for a week. The solution in the dialysis bag was transferred into a large flask and vacuum dried at 40°C. The GO sheet in dark color was obtained, dried and properly stored. 0.1 g of GO was weighed, dissolved in 100 ml water and ultrasonically treated. Centrifugation was performed at 8000 r/min to remove the precipitate. The yellowish brown supernatant was 1 g/L GO solution, which was preserved at 4°C in a fridge.

Fluorescence Quenching by Gradient GO

Serial dilution was performed over the above GO solution. Then 120 μL of 1 μM DNA5 and DNA6 was taken and properly mixed, and 40 μL of the solution was added with 60 μL water and 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.02 g/L and 0.01 g/L GO, respectively. Two parallels were set. After oscillation at 37°C for 2h, the fluorescence intensity was measured using F-4600 fluorescence spectrophotometer (voltage 700V, slit width 10 nm, scan rate 1200 nm/s, excitation light 470 nm and 630 nm, corresponding to FAM and Cy5, respectively). The curve of fluorescence intensity vs. GO concentration was plotted. GO concentration was selected as 0.1 g/L in the following experiment.

RNA Detection

200 μL of carboxyl-modified magnetic microspheres (MB-COOH) was taken and washed with 400 μL 0.1 M imidazole-HCL buffer (pH 6.8) three times, followed by washing with 400 μL 0.01 M phosphate buffered saline (PBS) (pH 7.4) three times. After magnetic separation, the supernatant was removed and 200 μL PBS was added. 100 μL activation solution (EDC: 4 mg/ml, NHS: 6 mg/ml) was added for activation at 37°C in a gas bath thermostatic oscillator. After washing with PBS three times, 200 μL 10^{-6} M amino-modified DNA, DNA1 and DNA2 were added, respectively, followed by oscillation at 37°C for 2h. The solution was washed with PBS three times, and the precipitate was collected and added with 200 μL 10^{-6} M DNA3 and DNA4, respectively. This was fol-

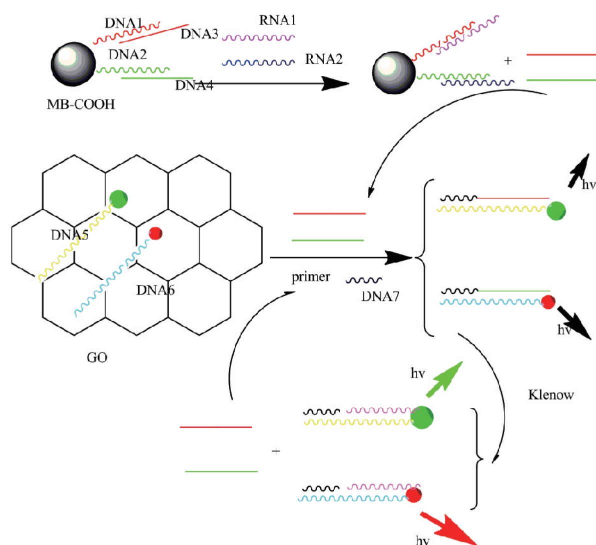

Figure 1. Multiple fluorescence detection based on GO.

lowed by oscillation at 37°C for 2h and washing with PBS three times with supernatant discarded. Water was added to reach the final volume of 750 µL. 50 µL was taken with 3 parallels, and 50 µL of RNA1 and RNA2 with different concentration was added, respectively. After oscillation at 37°C for 2h, 80 µL supernatants was collected.

Fluorescence detection of different concentration of RNA, 300 µL 1 µM DNA5 was mixed with 300 µL 1 µM DNA6, and 300 µL 0.1 g/L GO was added for treatment for 10 min. The solution was equally divided into 15 parts (60 µL each part). Then 300 µL 10 µM DNA7, 270 µL 2 mM dNTP, 300 µL polymerase buffer (10 times concentration) and 30 µL polymerases were mixed together. Into each part (60 µL) 15 samples were added, and into 15 samples, 80 µL of the above supernatant was added with proper mixing. After reaction at 37°C in the gas bath thermostatic oscillator for 2h, the fluorescence intensity was measured using F-4600 fluorescence spectrophotometer (voltage 700V, slit width 10 nm, scan rate 1200 nm/s, excitation light 470 nm and 630 nm, corresponding to FAM and Cy5, respectively). Using the detected signals, the concentration curve and the working curve were plotted.

Results

Principle

As shown in Figure 1, the carboxyl-modified magnetic microspheres bind to the amino-modified DNA1 and DNA2 via the amide bond; the microspheres are hybridized with the mismatched DNA3 and DNA4. The added RNA1 and RNA2 are completely complementary with the DNA1 and DNA2 on the microspheres and competitively substitute the bonded DNA1 and DNA2.

Characterization of GO

TEM of GO is shown in Figure 2. GO exists as thin sheets with folds, indicating the synthesis of GO from graphite powder and the subsequent exfoliation.

Fluorescence Quenching by Gradient GO Solution

The fluorescence intensity was measured, and the plots were drawn as shown in Figure 3. Solutions of different DNA sequences labeled with different fluorescent dyes (equal amount and equal concentration) were combined and added to gradient GO solutions. Two fluorescent dyes, FAM and Cy5, were excited at the wavelength of 470 nm and 630 nm, respectively. The maximum emission wavelength was 520 nm and 670 nm, respectively. The fluorescence was completely quenched at GO concentration of about 0.1 g/L.

Fluorescence Detection of two RNA Sequences

The fluorescence intensity was plotted for RNA1 and RNA2 as shown in Figure 4 and Figure 5, respectively. The signal intensity of both FAM and Cy5 increased with the increase of RNA concentration, showing a good linear relationship.

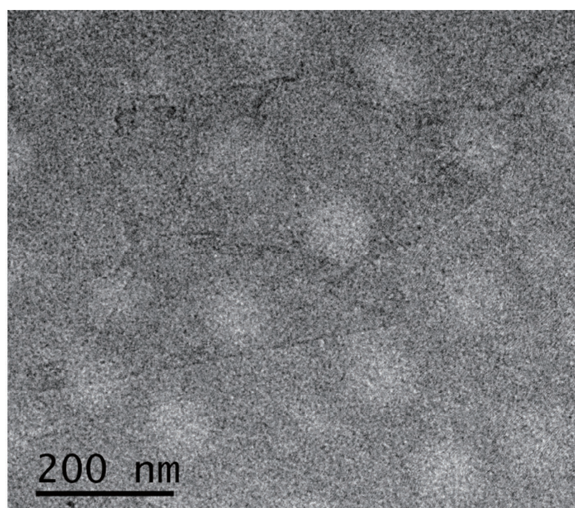

Figure 2. The transmission electron microscope (TEM) image of GO.

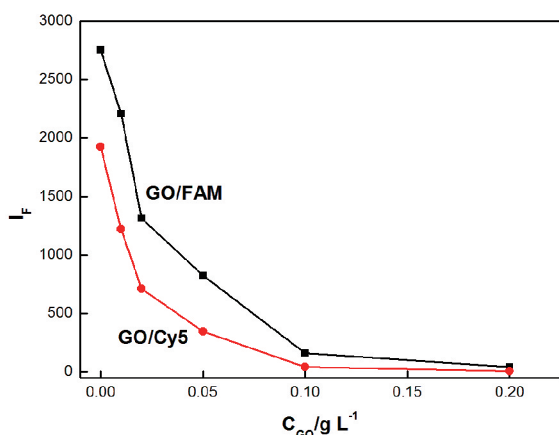

Figure 3. Fluorescence quenching by gradient GO.

The working curve of fluorescence intensity vs. RNA concentration was also plotted. For RNA1, the lowest limit of detection was 1.0×10^{-11} M and the response of fluorescent signal intensity was linear within the concentration range of 1.0×10^{-11} M to 1.0×10^{-9} M; the regression equation was Y = 28.6 X + 13.03 (Y is relative fluorescence intensity; X is concentration of RNA1, 10^{-10} M; R = 0.99). For RNA2, the lowest limit of detection was also 1.0×10^{-11} M, and the response of fluorescent signal intensity was linear within the concentration range of 1.0×10^{-11} M to 1.0×10^{-9} M; the regression equation was Y = 29.9 X + 8.56 (Y is relative fluorescence intensity; X is concentration of RNA2, 10^{-10} M; R = 0.99).

Discussion

The present study showed that GO will adsorb FAM- and Cy5-labeled single-stranded DNA5 and DNA6, respectively, while the substituted DNA3 and DNA4 will form double strands with DNA5 and DNA6 in the solution. Primer is then extended using the Klenow fragment and DNA3 and DNA4 are released back to the solution. Thus, the cyclic utilization of DNA3 and DNA4 is realized. As more and more DNA5 and DNA6 are released back to the solution, the fluorescence signals are amplified, based on which two RNA sequences can be detected. GO exists as thin sheets with folds, indicating the synthesis of GO from graphite powder and the subsequent exfoliation. Moreover, as the GO concentration increased, the fluorescent intensity decreased significantly with two dyes.

The results showed that the fluorescence was completely quenched at GO concentration of about 0.1 g/L. Thus, this concentration and the corresponding concentration of fluorescent DNA were used for the subsequent experiment. With the fluorescence detection of two RNA sequences, two RNA sequences were successfully detected by labeling the terminals of DNA with different fluorescent dyes. There is no need for separation and purification of the product before analysis. And the working curve of fluorescence intensity vs. RNA concentration was also plotted. The study demonstrated that the method was capable of detecting low concentration of two RNA sequences. Multiple RNA sequences can be detected by

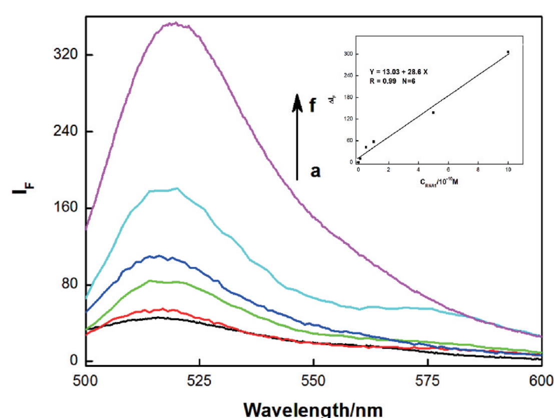

Figure 4. (A) Fluorescence intensity responses with different target RNA1 concentrations a-f: 0, 1.0×10^{-11}, 5.0×10^{-11}, 1.0×10^{-10}, 5.0×10^{-10}, 1.0×10^{-9} M). Inset: a calibration plot of saturated fluorescence intensity shift versus RNA1 concentration.

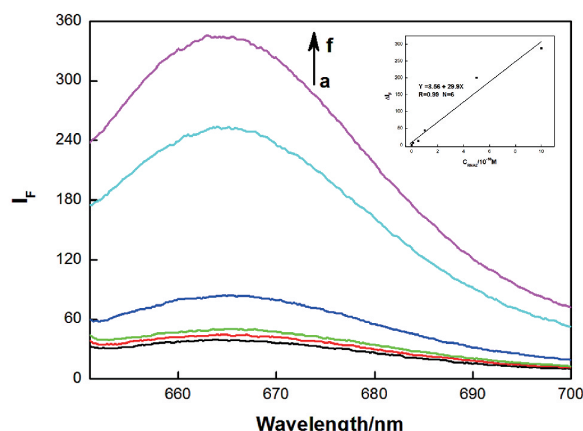

Figure 5. (A) Fluorescence intensity responses with different target RNA1 concentrations a-f: 0, 1.0×10^{-11}, 5.0×10^{-11}, 1.0×10^{-10}, 5.0×10^{-10}, 1.0×10^{-9} M). Inset: a calibration plot of saturated fluorescence intensity shift versus RNA2 concentration.

labeling the terminal of DNA with different fluorescent dyes.

Graphene and GO were first reported in 2004. Since then they are widely applied to various fields because of their unique physical and chemical properties. The nucleic acid binding ability of GO can help prevent the cleavage of DNA. GO can protect the RNA probes from the attack by the nuclease in the environment and thus stabilize RNA during the detection, enrichment and separation of the targets. Furthermore, GO can adsorb ssDNA or deadsorb dsDNA, thus achieving protection or deprotection. By utilizing this feature, cyclic enzymatic amplification method (CEAM) is developed for high-sensitivity detection of the targets. The sensitivity using CEAM can be improved by 2-3 orders of magnitude compared with conventional methods.

Conclusions

We prepared GO in this study and designed the polymerase-assisted cyclic amplification method based on the adsorption ability of GO on single-stranded DNA and FRET. By detecting the fluorescence signals, two RNA sequences were detected without the need for separation and purification before analysis. The lower limit of detection with this method is 1.0×10^{-11} M for different RNA, and the response of fluorescence intensity is linear within the concentration range of 1.0×10^{-11} M to 1.0×10^{-9} M. This method based on fluorescence detection is simple and fast, which has high sensitivity. It is suitable for detecting many specific RNA sequences in osteosarcoma cells with less damage to the patients and higher detection rate and accuracy rate.

Conflicts of interest
The authors declare no conflicts of interest.

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