Analytica Chimica Acta xxx (2016) 1–8

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

# A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles

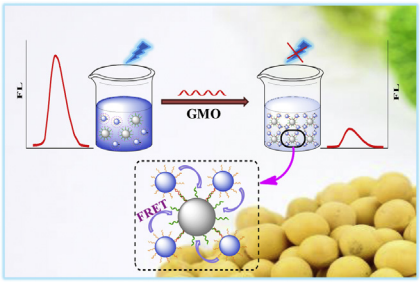
Yaqi Li [a], Li Sun [a], Jing Qian [b], Chengke Wang [a], Qian Liu [b], En Han [a], Nan Hao [b], Liuping Zhang [c], Jianrong Cai [a, **], Kun Wang [b, *]

[a] School of Food and Biological Engineering, Jiangsu University, Zhenjiang, 212013, China
[b] School of Chemistry and Chemical Engineering, Jiangsu University, Zhenjiang, 212013, China
[c] Sinograin Zhenjiang Grains & Oils Quality Testing Center Co., Ltd., Zhenjiang, 212013, China

## HIGHLIGHTS

- Both NGQDs and AgNPs were selected as the novel FRET donor-acceptor pairs.
- The proposed homogeneous FRET assay was developed for CaMV35S detection.
- The resulting method could identify 0.5% containing transgenic soybean sample.
- This assay was inexpensive, simple and highly sensitive.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 28 August 2016
Received in revised form
9 October 2016
Accepted 18 October 2016
Available online xxx

*Keywords:*
Nitrogen-doped graphene quantum dots
Silver nanoparticles
Fluorescence resonance energy transfer
Homogeneous assay
Transgenic soybean

## ABSTRACT

In this work, a novel homogeneous assay for DNA quantitative analysis based on förster resonance energy transfer (FRET) was developed for cauliflwer mosaic virus 35s (CaMV35S) promoter of transgenic soybean detection. The homogenous FRET of fluorescence signal was fabricated by DNA hybridization with probe modified nitrogen-doped graphene quantum dots (NGQDs) and silver nanoparticles (AgNPs), which acted the donor-acceptor pairs for the first time. The highly efficient FRET and unique properties of the NGQDs made the proposed FRET system as a functionalized detection platform for labelling of DNA. Upon the recognition of specific target DNA (tDNA), the FRET between NGQDs and AgNPs was triggered to produce fluorescence quenching, which could be used for tDNA detection. The fabricated homogeneous FRET assay displayed a wide linear range of 0.1−500.0 nM and a low limit of detection 0.03 nM for the detection of CaMV35S (S/N = 3). This proposed biosensor revealed high specificity to detect tDNA, with acceptable intra-assay precision and excellent stability. This method was successfully applied to identify the real sample of 0.5% containing transgenic soybean, which achieved the most of national law regulations. This assay was further validated by polymerase chain reaction as the genetically modified organisms, suggesting that the proposed FRET system is a feasible tool for the further daily genetically modified organism detection.



* Corresponding author.
** Corresponding author.
*E-mail addresses:* jrcai@ujs.edu.cn (J. Cai), wangkun@ujs.edu.cn (K. Wang).

http://dx.doi.org/10.1016/j.aca.2016.10.031
0003-2670/

## 1. Introduction

Genetically modified organisms (GMOs) have brought certain benefits in agriculture field due to their desirable traits including herbicide tolerance, virus resistance, delayed ripening, insect resistance, disease tolerance and improvement of nutritional content [1], and the area cultivated with GMOs increased nearly to 181.5 million hectares in 2014 [2]. Transgenic soybean is the most important GMOs, which was developed since the 1980, plays a very crucial role in alleviating of poverty and hunger and bringing great benefits [3,4]. However, the long term effects of transgenic soybean on human beings and the global ecology remain uncertain. These modified foods and feeds have not gained worldwide acceptance because consumers mistrust resulting from environmental and public health safety concerns [5,6]. As a result of these international concerns about possible potential risks, the adequate management of transgenic soybean and their products is of utmost importance. The labeling of transgenic soybean has been mandated in many nations, such as the European Union, Korea, Japan, and Australia. According to the labeling regulations, the threshold level of unintended transgenic components in food products is 0.9~5% [7]. Therefore, searching for reliable analytical methods for transgenic soybean detection has become increasingly important subject. During the past two decades, there have been some analytical methods developed, including polymerase chain reaction (PCR) [8], quartz crystal microbalance [9], surface plasmon resonance biosensor [10], etc. While all these methods have been already available performance the detection of transgenic soybean, they have one or more shortcomings, such as complex, time consuming, laborious and costly [11]. Consequently, it is still of critical urgency and necessary to fabricate the inexpensive, simple, highly sensitive, and convenient method for detecting of transgenic soybean.

Homogeneous assays are normally performed in a single reaction system, possessing many advantages such as biochemical universality, inexpensive experiment condition, operational convenience, robustness and naturally exceptional selectivity [12–15], especially, separation free, less tedious to performance than heterogeneous assay [16]. Förster resonance energy transfer (FRET) is a common technique used in homogeneous assays due to its equipment simplicity, high sensitivity, less sample required rapid analysis, and so on [17]. FRET based homogeneous assays are widely used for to detect target in the field of hazardous articles [18], biomolecules [19], living cells [20]. During the process of the homogeneous FRET assay fabrication, it is the core of the FRET technique to select the appropriate fluorescence resonance energy donor-acceptor pairs, which should match two important conditions: (1) the spectral overlap between the emission of the donor and the adsorption spectrum of the acceptor is greater than 30%; (2) the distance of the donor and the acceptor must be less than 10 nm [21]. Following this strategy, a series of appropriate donor-acceptor pairs have been fabricated including CdS/ZnS quantum dots(QDs)-organic fluorophore Cy5 [21], cyan fluorescent protein-yellow fluorescent protein [22] and CdS QDs-Au nanoparticles [23], graphene QDs (GQDs)-Au nanoparticles [24], etc. Recent studies have found that some assays are not flawless, for instance, the low chemical stability and photobleaching of typical organic dyes deteriorated the stability of these sensors [13], and the toxicity of the semiconductor QDs remains a subject of discussions [25]. Therefore, it is necessary to develop the suitable donor-acceptor pairs which are nontoxic, good stability and simple operation for the homogeneous FRET assay.

More recently, nitrogen-doped GQDs (NGQDs) have gotten significant attention, as a result of that, compared with the semiconductor QDs containing toxicity heavy metal constituents, doping chemically-bonded N atoms can offer more active sites, effectively tune their optical property, advantage the properties of low toxicity, simple synthesis process and good biocompatibility [25,26]. Therefore, NGQDs have been employed in various fields such as fuel cells [27,28], biosensor [29], and the detection of heavy metal ions and small molecules [26,30]. In particular, the studies concerning the target detection using the fluorescence (FL) property of NGQDs have attracted increasing interest due to the high sensitivity and selectivity of the FL technique [25,31,32]. Meanwhile, silver nanoparticles (AgNPs) could be applied as efficient acceptors due to their broad absorption band and the high extinction coefficient [33,34]. It's worth noting that the UV–vis absorbance peak of AgNPs is about at 405 nm [35], and according to our previous research, the maximum FL emission spectrum of the NGQDs is 435 nm [36], what shows good spectral overlap. In consequence, it is feasible to fabricate the homogeneous FRET assay based on NGQDs-AgNPs as donors and acceptors, respectively.

In this study, for the first time, a novel homogeneous FRET assay, with NGQD and AgNP as the fluorescence resonance energy donor-acceptor pairs (40% spectral overlap), was developed for transgenic soybeans gene of cauliflwer mosaic virus 35s (CaMV35S) promoter detection. This assay was fabricated by DNA hybridization with probe modified NGQDs and AgNPs. The highly efficient FRET and unique properties of the NGQDs make the proposed FRET system as a functionalized detection platform for labelling of DNA. Upon the recognition of specific target DNA (tDNA), the FRET between NGQDs and AgNPs was triggered to produce fluorescence quenching, which could be used for target DNA detection. This proposed homogeneous FRET assay was utilized for the sensitive and selective detection of CaMV35S promoter. Various parameters which could influence the homogeneous FRET assay including reactant ratio of donor to acceptor, pH value of buffer, buffer ionic strength and stability were systematically optimized. This assay was successfully applied to identify the real sample of 0.5% containing transgenic soybean, what confirmed with most of national law regulations. The approach was further validated by PCR as the GMOs target. It was demonstrated this method was utility and reliable for quantification of GMOs in food.

## 2. Experimental

### 2.1. Apparatus

The absorption spectra were measured on a UV-2450 spectrophotometer (Shimadzu, Japan). FL spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan). The transmission electron microscopy (TEM) images were taken by a JEOL 2100 TEM (JEOL, Japan) at 200 kV. The X-ray photoelectron spectroscopy (XPS) data were obtained by an Escalab-MKII spectrometer with Mo Kα X-rays as the excitation source.

### 2.2. Materials

Ammoniumcitrate (AC), silver nitrate ($AgNO_3$), sodium borohydride was provided by Sinopharm Chemical Reagent Co., Ltd. 1–Ethyl–3–(3–dimethyl–aminopropyl) carbodiimide (EDC) and N–hydroxysuccinimide (NHS) was purchased from Sigma Aldrich. Different kinds of buffers such as 10.0 M Tris-HCl (pH 8.0) and 1 × TAE buffer (40.0 M Tris, 1.0 M EDTA, 40.0 M acetate, pH 8.0) were used in the experiment. All the reagents were of analytical reagent grade and doubly distilled water was used throughout the experiments. CaMV35S promoter was used as the target nucleotides sequences. All synthetic oligonucleotides were obtained from Sangon Biotech Co., Ltd. (Shanghai, China) and listed in Table 1.

All oligonucleotides were dissolved in Tris-HCl to prepare stock solution. The real samples containing the CaMV35S promoter was

Please cite this article in press as: Y. Li, et al., A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles, Analytica Chimica Acta (2016), http://dx.doi.org/10.1016/j.aca.2016.10.031

extracted from transgenic soybean using an Ultra Clean TM Microbial DNA Kit (Sangon Biotech Co., Ltd). The PCR amplification target gene was performed with primers of forward and reverse.

### 2.3. Preparation of AgNPs and AgNPs-probe$^{II}$

AgNPs with average size of 12 nm were prepared by reduction of $AgNO_3$ with sodium borohydride in water [34]. Briefly, ice cold $AgNO_3$ (2.0 mM) and two times volume of $NaBH_4$ (3.0 mM) was mixed dropwise with vigorously stirring in an ice bath, and the mixture was continuously stirred until the temperature naturally increased to the room temperature. The probe$^{II}$ was modified on the surface of AgNPs according to the previous report [37]. Briefly, 1 mL of AgNPs solution with probe$^{II}$ (100 μL, 10.0 μM) was incubated for at least 20 h. To adjust the pH value and increase ionic strength of the resulting solution, 125 μL of 10.0 mM Tris-HCl was added to the solution and allowed to react for 6 h. Then 20 μL of 2.0 M NaCl was added to the solution and this procedure was repeated two times at the interval of 3 h in order to make the total NaCl concentration increasing gradually to 2.0 M. After an additional standing for 48 h at least, the AgNPs-probe$^{II}$ were isolated by centrifugation for 10 min, 3 times at 15000 rpm. And then the resulting AgNPs-probe$^{II}$ conjugate precipitate was washed and redispersed in 10.0 mM Tris-HCl.

### 2.4. Preparation of NGQDs and NGQDs-probe$^{I}$

The NGQDs with average size of 5 nm was prepared in our previous work [36], 0.5 g AC and 15 mL $H_2O$ were put into a three-necked flask and heated to 200 °C using an oilbathpan in the closed environment. The whole process was carried out under the condition of reflux. Subsequently, the color of the liquid was changed from colorless to pale yellow, and then orange in 0.5 h, suggesting the formation of NGQDs. Ultimately, The pH value of obtained aqueous solution was adjusted to 8.0 by the addition of NaOH solution and the NGQDs solution was stored at 4 °C for later use. The prepared NGQDs solution was firstly sonicated for 15 min. EDC (40.0 mM) solution was then added into the NGQDs suspensions. The mixture was shaken on a vortex mixer for 2 min and bath sonicated for another 15 min. After that, probe$^{I}$ was added into the mixture and incubated for 40 min at room temperature. The final products of the NGQD-probe$^{I}$ conjugate were obtained by centrifugation and washing with PBS buffer three times to remove the free non-conjugated complex.

### 2.5. Procedure of homogeneous FRET assay

To investigate the effect of tDNA concentrations on FL quenching efficiency, the procedure of FRET quenching was performance as followed. The NGQDs-probe$^{I}$ was incubated with different tDNA concentrations (0–500.0 nM) for 2 h to allow hybridization at room temperature. Subsequently, 50 μL probe$^{II}$ modified AgNPs solution was mixed with 50 μL of the above mixture solution for incubation of 2 h at room temperature to form the co-hybridized sandwich complex structure. After incubation, the FL intensity was measured. All the experiments were measured under the same condition. The specificity of the system was also evaluated with 2-base mismatched tDNA, 5-base mismatched tDNA.

**Table 1**
Sequences of oligonucleotides used in this work.

| Name | Sequence (from 5′ to 3′) |
|---|---|
| CaMV35S target DNA | GGCAGAGGCATCTTCAACGATGGCC |
| Amine-modified probe (probe$^{I}$) | GAT GCC TCT GCC-$NH_2$ |
| Thiol-modified probe (probe$^{II}$) | HS-G GCC ATC GTT GAA |
| forward primer CaMV35S | GCTCCTACAAATGCCATCA |
| reverse primer CaMV35S | GATAGTGGGATTGTGCGTCA |
| 2-base mismatched target DNA | GGCAGTGGCATCTTCAATGATGGCC |
| 5-base mismatched target DNA | GGTAGAGTCATCTGCAACTATGGAC |

### 2.6. Detection of CaMV35S in transgenic soybean samples

The samples were extracted from transgenic soybean powder according to the manufacturer's instructions of the Plant Genomic DNA Kit, which were diluted with double-distilled water to the various concentrations of containing 0.5%, 1.0% and 10.0% GMOs. Subsequently, the serial dilutions of GMOs were amplified by the PCR method. Then 6 μL of the PCR products were analyzed by electrophoresis separation at 5 V/cm for 40 min on a 2% agarose gel, which contained 0.5 μg/mL of ethidium bromide in 1 × TAE buffer (pH 8.0). Finally, the PCR products were diluted with 10.0 mM Tris-HCL, denatured by heating in a boiling water bath for 5 min and then frozen in an ice bath for 1 min to obtain the single-stranded DNA solution [38]. The homogeneous FRET assay of the detection for CaMV35S was then applied to the real samples amplified by the PCR.

## 3. Results and discussion

### 3.1. Mechanism of homogeneous FRET assay

The fabricated procedure of the probes was shown in Fig. 1A, initially, amine modified capture probe (probe$^{I}$) were immobilized on the NGQDs surface through the reaction of activated carboxylic acid group and amino group, meanwhile AgNPs were conjugated with thiol-modified probe (probe$^{II}$) by the strong affinity of Ag–S bonding [39,40]. The detailed sensing mechanism of the FRET strategy of the proposed NGQDs-AgNPs for tDNA of CaMV35S detection is illustrated in Fig. 1B. As a result of that both NGQDs-probe$^{I}$ and AgNPs-probe$^{II}$ carried the negative charge (Supporting Information), they were not connected with each other when tDNA was absent. Meanwhile, the NGQDs-probe$^{I}$ had strong fluorescent emission. After tDNA of the CaMV35S co-hybridized with probe$^{I}$ on NGQDs and probe$^{II}$ on AgNPs, the emission of NGQDs was absorbed by AgNPs under the 355 nm light excitation. NGQDs and AgNPs acted as the donor and the acceptor of FRET pairs. The co-hybridized brought NGQDs and AgNPs into close proximity to occur the highly efficient FRET, which leaded to the decrease the intensity of fluorescent signal. Through comparing the fluorescent signal change, it was realized the monitoring of tDNA.

### 3.2. Characterization of NGQDs and AgNPs

The AgNPs were synthesized by the sodium borohydride reduction method. TEM experiment was performed to characterize the morphology and size of AgNPs. Fig. 2A is showing that the synthesized AgNPs had good round shape and uniform size with the average diameter of 12 nm. As shown in Fig. 2B the well-dispersed NGQDs with the average size of 5 nm can be observed. The HR-TEM result (the inset of Fig. 2B) indicated high crystallinity of the NGQDs with lattice spacing of 0.21 nm, which was accordant with the previous reports [41,42]. Fig. 2C presented the NGQDs dispersion had optimal maximum excitation and emission wavelengths at 355 nm and 435 nm, respectively. The photograph of the solution showed that the color of NGQDs was a yellow color under visible light, while no FL was observed and gives blue FL emission under the excitation by 365 nm UV light, further indicating the resulting NGQDs were fluorescent. Additionally, the uniform blue color distribution demonstrated that NGQD dispersed well in water

Please cite this article in press as: Y. Li, et al., A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles, Analytica Chimica Acta (2016), http://dx.doi.org/10.1016/j.aca.2016.10.031

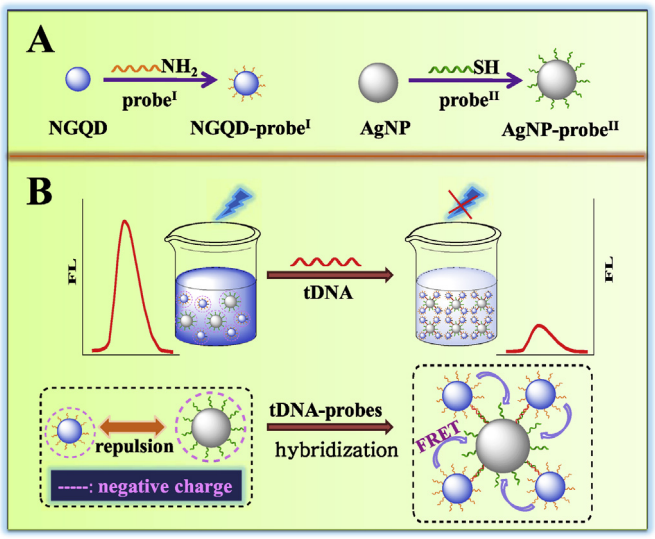



**Fig. 1.** (A) The schematic illustration of the probes fabrication; (B) the sensing mechanism of homogeneous FRET assay based on NGQDs and AgNPs for CaMV35S detection.

solution due to the abundant oxygen-containing functional groups on the surface. The emission wavelength of NGQDs was nearly excitation-independent, with the maximum excitation wavelength and the maximum emission wavelength at 355 and 435 nm, respectively (Fig. 2D). In addition, compared with GQDs, the FL emission of NGQDs was blue-shift, which was believed to be from the strong electron affinity of N atoms doped in the NGQDs [31,43]. All the results above demonstrated the successful preparation of NGQDs.

### 3.3. Spectral properties of NGQDs-probe$^{I}$ and AgNPs-probe$^{II}$

The spectral characteristics of donor and acceptor were studied, as they were important elements to the FRET process [44]. Fig. 3 showed the emission and absorption spectra of NGQDs and AgNPs synthesized in this work. It was shown that the maximum emission wavelength of NGQDs was 435 nm with a full width at half-maximum (FWHM) of about 55 nm. After conjugation with probe$^{I}$, there was a slight red-shift in the peak of emission spectrum of NGQDs (Fig. 3A). Meanwhile, to verify the assembly of NGQDs with probe$^{I}$, the UV–vis absorption spectra of probe$^{I}$, NGQDs and NGQDs-probe$^{I}$ were acquired for comparison in Fig. 3B. It was obvious that the probe$^{I}$ solution exhibited an absorbance peak located at 257 nm, which corresponded to the characteristic peak of DNA, generated from the absorption of purine and thymine bases. It could be seen that the pure NGQDs aqueous suspension showed the transverse peak at 335 nm. While probe$^{I}$ was connected to the NGQDs, the characteristic peak of probe$^{I}$ at 257 nm was appeared in the absorption spectrum of NGQDs-probe$^{I}$, and the longitudinal peak of NGQDs at 335 nm red-shifted to 340 nm, suggesting the successful loading of probe$^{I}$ on NGQDs surface. As shown in Fig. 3C, the UV–vis adsorption spectra of probe$^{II}$, AgNPs and AgNPs-probe$^{II}$ were obtained. It could be noted that AgNPs-probe$^{II}$ had two obvious absorbance peaks at around 257 nm and 402 nm. To investigate the possibility of the homogeneous FRET assay, we recorded the UV–vis absorption and FL emission spectra of the AgNPs-probe$^{II}$ and NGQDs-probe$^{I}$. As shown in Fig. 3D the well-overlapping spectra (40% spectral overlap) between FL emission spectra of NGQDs and absorption spectra of AgNPs provided the feasibility of producing an efficient FRET process as long as they are

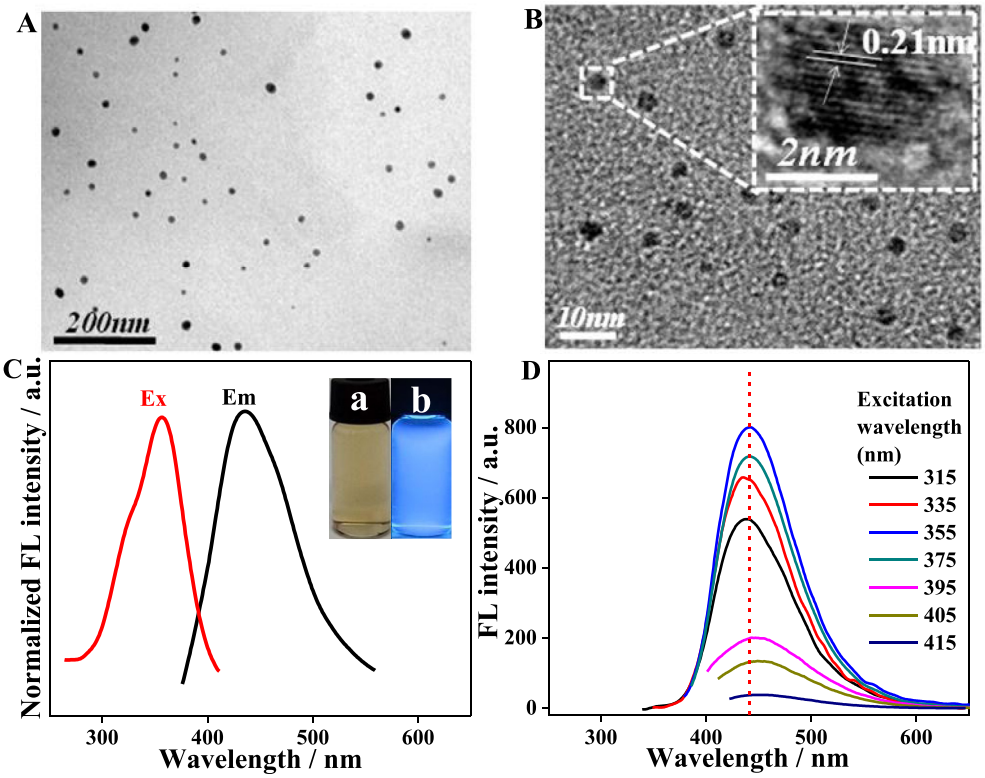



**Fig. 2.** TEM images of (A) synthesized AgNPs and (B) NGQDs, inset: HRTEM image of NGQDs; (C) normalized fluorescence spectrum of the NGQDs, inset: photographs taken under daylight (left) and 365 nm UV light (right); (D) fluorescence emission spectra of the NGQDs at different excitation wavelengths.

Please cite this article in press as: Y. Li, et al., A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles, Analytica Chimica Acta (2016), http://dx.doi.org/10.1016/j.aca.2016.10.031

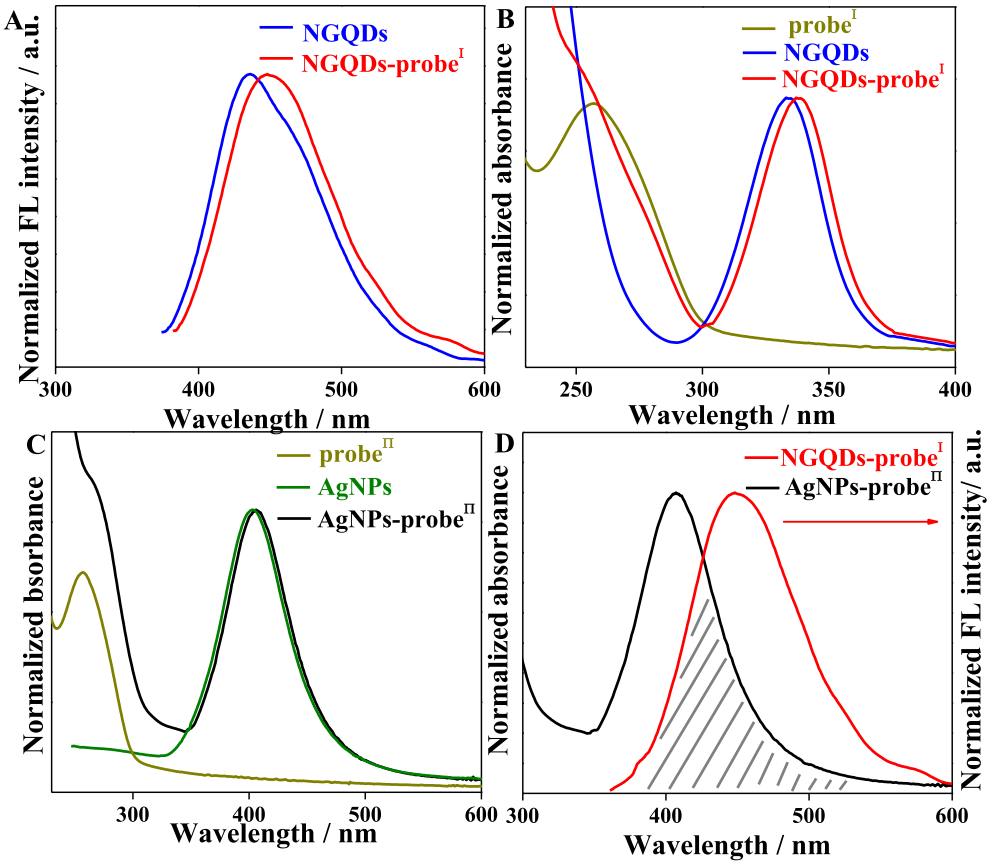



**Fig. 3.** (A) Normalized emission spectra of NGQDs before and after conjugation with probe$^{I}$; (B) normalized absorption spectra of probe$^{I}$, NGQDs and NGQDs-probe$^{I}$; (C) normalized absorption spectra of AgNPs before and after conjugation with probes; (D) spectra overlapping between normalized emission spectra of NGQDs-probe$^{I}$ and normalized absorption spectra of AgNPs-probe$^{II}$.

in a close proximity.

### 3.4. Stability of the NGQDs

The emission spectra of the as-prepared NGQD solution were measured under excitation at 355 nm with different placing time of 0, 60 and 120 min. As shown in Fig. S1 (Supporting Information), the FL intensity of NGQDs showed slight change. To confirm the stability of NGQDs under high-ionic-strength environments, the FL intensities were also recorded in the presence of different concentrations of NaCl (from 0.5 to 2.0 M). It is shown in Fig. S2 (Supporting Information), only a little change was observed, indicating that the high stability of NGQDs was obtained even under a high-ionic-strength environment. The result showed that NGQDs had great potential for sensing applications.

### 3.5. Optimization of conditions for CaMV35S promoter detection

To determine the optimum experimental conditions for tDNA detection, the pH value of the buffer solution is known being an important element for the FRET system, because unsuitable pH not only damage the probe, decrease the efficiency of hybridization, but also affect the affinity between probes and the NGQDs surface. The pH of the detection solution could also affect the FL property of NGQDs [24]. Therefore, the effect of pH value on the FL intensity of the FRET system was investigated in a series of pH values from 5.0 to 10.0 before the detection. The experimental results showed in Fig. S3 (Supporting Information), an increase in the pH value from 5.0 to 8.0 caused a remarkable increase in the FL quenching efficiency of NGQDs, but a further increase to 10.0 results in a gradual decrease of the FL quenching efficiency. Accordingly, the Tris-HCl buffer (10.0 mM) of pH 8.0 was used as the buffer with the optimum pH for tDNA detection. Furthermore, the effect of molar ratio of AgNPs-probe$^{II}$/NGQDs-probe$^{I}$ of the homogeneous FRET assay was also investigated by tuning the molar ratio of AgNPs-probe$^{II}$ to NGQDs-probe$^{I}$. Each data point was an average of six replicate measurements and the error bars correspond to the standard deviation of each measurement. As shown in Fig. 4A, FRET efficiencies were increasing when the ratio of AgNPs-probe$^{II}$ to NGQDs-probe$^{I}$ was at 1.5:1 and 1.9:1, respectively, then reached to the maximum quenching efficiency ca. 86% at the ratio of 2.3:1. After that, FRET efficiencies were obviously decreasing. As a result of that 2.3:1 was selected to be the optimal molar ratio, under these conditions, the hybrid complex of NGQDs-dsDNA-AgNPs possesses weak FL, which is favorable for the tDNA detection in bioanalysis.

### 3.6. Construction of homogeneous FRET assay for tDNA detection

Detection of complementary tDNA using such NGQDs-probe$^{I}$ conjugate was carried out through hybridization reaction in the presence of tDNA and AgNPs-probe$^{II}$ to form FRET assembly. To explore the quenching efficiency of this homogeneous FRET assay, a series of concentrations of tDNA were tested. As a result of sandwich assay structure formation of NGQDs-dsDNA-AgNPs, NGQDs were brought close to AgNPs surface and FL quenching was realized. Fig. 4B shows that the FL intensity centered at 435 nm decreased along with the increasing tDNA concentration, indicating that the addition of tDNA can effectively quench the FL of NGQDs.

Please cite this article in press as: Y. Li, et al., A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles, Analytica Chimica Acta (2016), http://dx.doi.org/10.1016/j.aca.2016.10.031

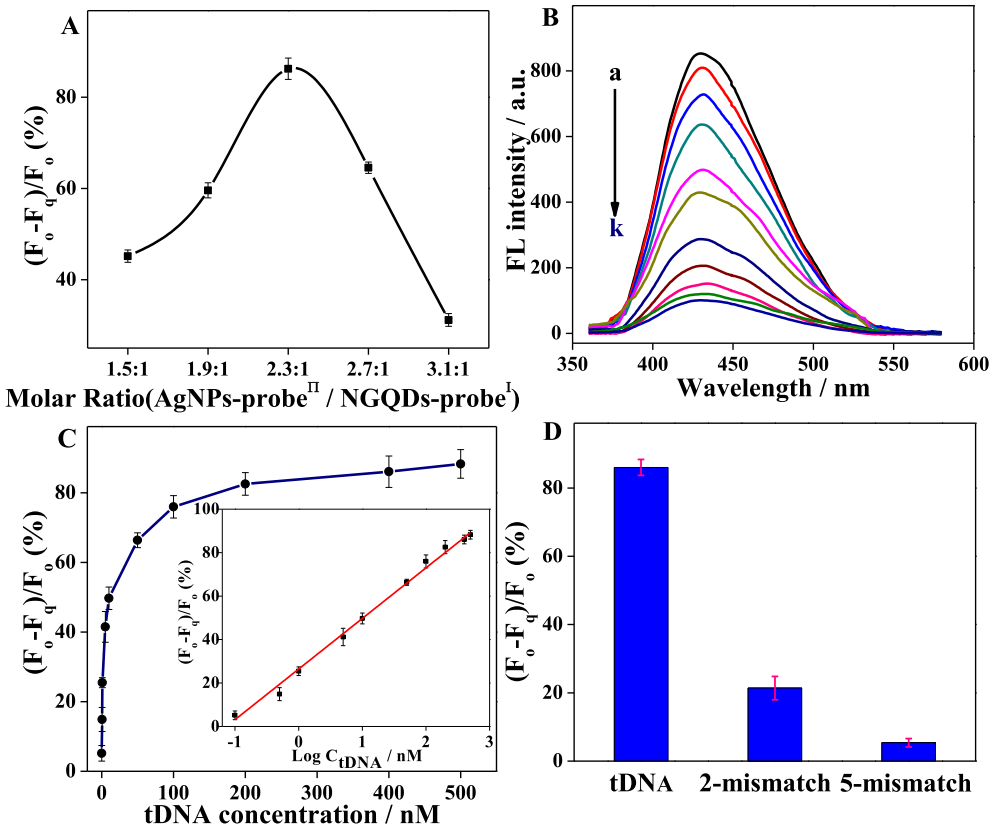



**Fig. 4.** (A) The effects from molar ratio of AgNPs-probe$^{II}$ to NGQDs-probe$^{I}$ for the fluorescent signal quenching efficiency; (B) Fluorescence spectra of the homogeneous FRET assay in the presence of target CaMV35S with different concentrations (0, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 200.0, 400.0, 500.0 nM); (C) The quenching efficiency $Q = 1-F_q/F_o$ versus with a series of target CaMV35S concentrations. Inset graph showed the quenching efficiency of fluorescence intensity varied with logarithm of concentration of target CaMV35S; (D) Quenching efficiency comparison among 2-base mismatched tDNA, 5-base mismatched tDNA, and Complementary target DNA with concentration of 200.0 nM.

The quenching efficiency was expressed by $Q = 1 - F_q/F_o$, where $F_q$ was the FL intensity of NGQDs after quenching, and $F_o$ was the original FL intensity of NGQDs. Fig. 4C presented the FL intensity versus the concentration of tDNA. The quenching efficiency gradually increased with the growing concentrations of tDNA and reached a value of 87% with 500 nM tDNA. A linear calibration plot in the concentration range of 0.1–500 nM with a correlation coefficient of 0.994 was displayed and the limit of detection (LOD) was down to 0.03 nM (S/N = 3). As shown in Table 2, compared with other reported detection methods, this method possessed a comparable linear range and a lower detection limit.

### 3.7. Specificity, reproducibility and stability of the NGQDs-dsDNA-AgNPs homogeneous FRET assay

To explore the specificity of the NGQDs-dsDNA-AgNPs FRET system, both 2-mismatching and 5-mismatching base pairs were used to compare with complementary tDNA sequences. All the experiments used the same concentration of 200 nM for comparison. As can be seen from Fig. 4D, the quenching efficiency decreased significantly with the increasing number of mismatches. The quenching efficiency for complementary tDNA was around 82%, while that of 2-mismatching and 5-mismatching base pairs dropped to 22% and 9% respectively. Generally, an increasing number of mismatches of tDNA would lead to decreasing duplex stability, which decreased the chances to form NGQDs-dsDNA-AgNPs sandwich complex and NGQDs emission would not decrease obviously [45]. The results indicated that this homogeneous FRET assay exhibited high selectivity. The reproducibility of this assay was estimated at the tDNA concentration of 200 nM, the relative standard deviation (RSD) corresponded to six independent experiments was calculated to be 3.6%, illustrating the proposed assay possessing excellent reproducibility. Meanwhile, the stability of the probe was also measured during storage in state at 4 °C. It was shown that the fluorescence intensity for 200 nM tDNA remained nearly 95.7% of its initial value after one month. The long-term stability could be attributed to good biocompatibility of probe and strong interaction of DNA specific hybridization. This illustrated the presented assay was feasible to monitor the tDNA with the advantages of high selectivity, excellent reproducibility and

**Table 2**
Different methods for determination of target DNA.

| FRET system | Linear range (nM) | LOD (nM) | Reference |
|---|---|---|---|
| CPEs[a]-GO[b] | 0.5–20.0 | 0.05 | [48] |
| AgNCs[c]-MWCNTs[d] | 31.0–2000.0 | 24.0 | [49] |
| GQDs[g]-CNs[h] | 1.5–133.0 | 0.4 | [50] |
| CdTe QDs[e]-EB[f] | 7.7–61.6 | 7.7 | [51] |
| CdSe/ZnS QDs-ROX[i] | 2.5–30.0 | 1.5 | [52] |
| NGQDs-AgNPs | 0.1–500.0 | 0.03 | This work |

[a] π-conjugated polyelectrolytes.
[b] Graphene oxide.
[c] Silver nanoclusters.
[d] Multi-walled carbon nanotubes.
[e] Quantum dots.
[f] Ethidium bromide.
[g] Graphene quantum dots.
[h] Carbon nanotubes.
[i] 6–carboxy–X–rhodamine.

Please cite this article in press as: Y. Li, et al., A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles, Analytica Chimica Acta (2016), http://dx.doi.org/10.1016/j.aca.2016.10.031

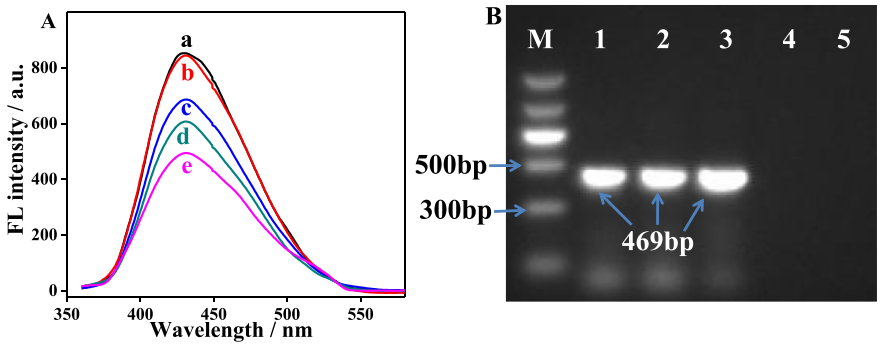



**Fig. 5.** (A) Emission spectra of the FRET system detection of (a) blank control, (b) non–genetically modified organisms (GMOs), (c) 0.5% containing GMOs, (d) 1.0% containing GMOs and (e) 10.0% containing GMOs; (B) Detection of the GMOs tDNA (CaMV35S) by 2% agarose gel electrophoresis. (M) DNA markers, (1) 0.5% GMOs, (2) 1.0% GMOs, (3) 10.0% GMOs, (4) non-GMOs, (5) blank control.

good stability.

### 3.8. Detection of PCR products of soybean

It was reported that PCR amplified real sample of soybean samples was further conducted by fabricated biosensors [46,47]. In this work, the various concentrations samples of containing 0%, 0.5%, 1% and 10% transgenic soybean were amplified by the PCR method, which were further detected by this FRET system with an excitation wavelength of 355 nm under the selected conditions. Fig. 5A showed the emission spectra of FRET detection blank control, the PCR product of non-transgenic and containing 0.5%, 1% and 10% of transgenic soybean samples. The homogeneous FRET assay performed excellent strong fluorescent intensity. While the concentration containing of transgenic soybean increased, the fluorescent intensity decreased. As demonstrated by gel electrophoresis analysis in Fig. 5B, the bands of CaMV35S (469bp) appeared in the lanes of containing 0.5%, 1% and 10% transgenic soybean clearly, while no bands were found in non-transgenic soybean or blank control. This result was consistent with the result detected by the FRET system, demonstrating a remarkable accuracy of the homogeneous FRET assay.

## 4. Conclusion

In summary, a homogeneous FRET assay based on NGQDs and AgNPs pairs (40% spectral overlap) was fabricated for CaMV35S specific gene detection. In this homogeneous FRET assay, NGQDs and AgNPs were the fluorescence resonance energy donor-acceptor pairs, respectively. This FRET platform was realized by immobilization of the amino-functionalized probe on NGQDs and conjugation of the thiol-functionalized probe on AgNPs. The sandwich structure formation was created by co-hybridization of tDNA with probe$^{I}$ and probe$^{II}$ brought NGQDs and AgNPs into close proximity to trigger FRET phenomena. In this study, a short gene sequence of CaMV35S was used as tDNA. The quenching efficiency was measured with a series of tDNA concentrations and the results demonstrated the feasibility of this homogeneous FRET assay for CaMV35S promoter detection with LOD of 0.03 nM. The experiments with 2-base mismatched DNA and 5-base mismatched DNA demonstrated the good selectivity. And the homogeneous FRET assay was used to detect the containing 0.5%, 1% and 10% transgenic soybean samples, the result demonstrated that the FRET system detection of 0.5% transgenic soybean with PCR-amplified can be achieved, which had the potential to be used as a sensitive, simple and portable platform for in-field of food safety and environmental screening.

## Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (Nos. 21375050, 21405063, 21505055 and 21675066), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (No. PAPD-2014-37), Senior Professional Research Start-up Fund of Jiangsu University (No. 5503000011), Major Project Supported by the Natural Science Foundation of the Jiangsu Higher Education Institutions, China (Grant No.15KJA550001), Innovation Project of Science and Technology for College Graduates of Jiangsu Province (No. KYLX15_1091), Project Funded by China Postdoctoral Science Foundation (No. 2015M581745 and No. 2015M580401), Project Funded by Jiangsu Province Postdoctoral Science Foundation (NO. 1501108C).

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.10.031.

## References

[1] S. Meriç, O. Cakır, N. Turgut, S. Arı, Detection of genetically modified maize and soybean in feed samples, Genet. Mol. Res. 13 (2014) 1160–1168.
[2] C. Manzanares, I. Mafra, J. Costa, M. Barroso, N. Álvarez, C. Delerue, M. Oliveira, M. Lobo, B. Ruiz, Electrochemical magnetoassay coupled to PCR as a quantitative approach to detect the soybean transgenic event GTS40-3-2 in foods, Sens. Actuators, B 222 (2016) 1050–1057.
[3] C.L. Manzanares-Palenzuela, N. De-Los-Santos-Álvarez, M.J. Lobo-Castañón, B. López-Ruiz, Multiplex electrochemical DNA platform for femtomolar-level quantification of genetically modified soybean, Biosens. Bioelectron. 68 (2015) 259–265.
[4] T. Yang, N. Zhou, Q. Li, Q. Guan, W. Zhang, K. Jiao, Highly sensitive electrochemical impedance sensing of PEP gene based on integrated Au-Pt alloy nanoparticles and polytyramine, Colloid. Surf. B 97 (2012) 150–154.
[5] B. Delaney, Safety assessment of foods from genetically modified crops in countries with developing economies, Food Chem. Toxicol. 86 (2015) 132–143.
[6] X. Huang, C.C. Zhai, Q.M. You, H.J. Chen, Potential of cross-priming amplification and DNA-based lateral-flow strip biosensor for rapid on-site GMO screening, Anal. Bioanal. Chem. 406 (2014) 4243–4249.
[7] F.G. Xing, W. Zhang, J.N. Selvaraj, Y. Liu, DNA degradation in genetically modified rice with Cry1Ab by food processing methods: implications for the quantification of genetically modified organisms, Food Chem. 174 (2015) 132–138.
[8] S.F. Rosa, F. Gatto, A. Angers-Loustau, M. Petrillo, J. Kreysa, M. Querci, Development and applicability of a ready-to-use PCR system for GMO screening, Food Chem. 201 (2016) 110–119.
[9] T.T.N. Lien, T.D. Lam, V.T.H. An, T.V. Hoang, D.T. Quang, D.Q. Khieu, T. Tsukahara, Y.H. Lee, J.S. Kim, Multi-wall carbon nanotubes (MWCNTs)-doped polypyrrole DNA biosensor for label-free detection of genetically modified organisms by QCM and EIS, Talanta 80 (2015) 1164–1169.
[10] P.D. Tam, Genetically modified organism (GMO) detection by biosensor based on SWCNT material, Curr. Appl. Phys. 15 (2015) 397–401.

[11] A. Ulianas, L.Y. Heng, M. Ahmad, H.Y. Lau, Z. Ishak, L.L. Tan, A regenerable screen-printed DNA biosensor based on acrylic microsphere-gold nanoparticle composite for genetically modified soybean determination, Sens. Actuators, B 190 (2014) 694–701.
[12] J.H. Han, L. Sudheendra, I.M. Kennedy, FRET-based homogeneous immunoassay on a nanoparticle-based photonic crystal, Anal. Bioanal. Chem. 407 (2015) 5243–5247.
[13] W. Xu, Y.H. Xiong, W.H. Lai, Y. Xu, C.M. Li, M.Y. Xie, A homogeneous immunosensor for AFB1 detection based on FRET between different-sized quantum dots, Biosens. Bioelectron. 56 (2014) 144–150.
[14] C.X. Tan, J.A. Schenk, N. Gajovic-Eichelmann, F. Sellrie, F. Bier, A new one-step antigen heterologous homogeneous fluorescence immunoassay for progesterone detection in serum, Talanta 134 (2015) 508–513.
[15] H. Li, X.E. Fang, H.M. Cao, J.L. Kong, Paper-based fluorescence resonance energy transfer assay for directly detecting nucleic acids and proteins, Biosens. Bioelectron. 80 (2016) 79–83.
[16] T. Kokko, L. Kokko, T. Soukka, T. Lovgren, Homogeneous non-competitive bioaffinity assay based on fluorescence resonance energy transfer, Anal. Chim. Acta 585 (2007) 120–125.
[17] Y.B. Miao, H.X. Ren, N. Gan, Y. Zhou, Y.T. Cao, T.H. Li, Y.J. Chen, A homogeneous and "off–on" fluorescence aptamer-based assay for chloramphenicol using vesicle quantum dot-gold colloid composite probes, Anal. Chim. Acta 929 (2016) 49–55.
[18] Y.Q. Yu, Q. Cao, M. Zhou, H. Cui, A novel homogeneous label-free aptasensor for 2,4,6-trinitrotoluene detection based on an assembly strategy of electrochemiluminescent graphene oxide with gold nanoparticles and aptamer, Biosens. Bioelectron. 43 (2013) 137–142.
[19] T.H. Li, J.Y. Byun, B.B. Kim, Y.B. Shin, M.G. Kim, Label-free homogeneous FRET immunoassay for the detection of mycotoxins that utilizes quenching of the intrinsic fluorescence of antibodies, Biosens. Bioelectron. 42 (2013) 403–408.
[20] Y. Liu, Z.M. Zhao, J.Y. Miao, B.X. Zhao, A ratiometric fluorescent probe based on boron dipyrromethene and rhodamine Förster resonance energy transfer platform for hypochlorous acid and its application in living cells, Anal. Chim. Acta 921 (2016) 77–83.
[21] M. Stanisavljevic, S. Krizkova, M. Vaculovicova, R. Kizek, V. Adam, Quantum dots-fluorescence resonance energy transfer-based nanosensors and their application, Biosens. Bioelectron. 74 (2015) 562–574.
[22] K.F. Chou, A. Dennis, Förster resonance energy transfer between quantum dot donors and quantum dot acceptors, Sensors 15 (2015) 13288–13325.
[23] L. Deng, Y. Du, J. Xu, H. Chen, One-pot green synthesis of oxygen-rich nitrogen-doped graphene quantum dots and their potential application in pH-sensitive photoluminescence and detection of mercury(II) ions, Biosens. Bioelectron. 59 (2014) 58–63.
[24] B.F. Shi, L.L. Zhang, C.Q. Lan, J.J. Zhao, Y.B. Su, S.L. Zhao, One-pot green synthesis of oxygen-rich nitrogen-doped grapheme quantum dots and their potential application in pH-sensitive photo-luminescence and detection of mercury(II) ions, Talanta 142 (2015) 131–139.
[25] C.Q. Wang, J. Qian, K. Wang, M.J. Hua, Q. Liu, N. Hao, T.Y. You, X.Y. Huang, Nitrogen-doped graphene quantum dots@$SiO_2$ nanoparticles as electrochemiluminescence and fluorescence signal indicators for magnetically controlled aptasensor with dual detection channels, ACS Appl. Mater. Interfaces 7 (2015) 26865–26873.
[26] J.Y. Shi, C.Y. Chan, Y.T. Pang, W.W. Ye, F. Tian, J. Lyu, Y. Zhang, M. Yang, A fluorescence resonance energy transfer (FRET) biosensor based on graphene quantum dots (GQDs) and gold nanoparticles (AuNPs) for the detection of mecA gene sequence of Staphylococcus aureus, Biosens. Bioelectron. 67 (2015) 595–600.
[27] Q. Liu, B. Guo, Z. Rao, B. Zhang, J.R. Gong, Strong two-photon-induced fluorescence from photo stable, biocompatible nitrogen-doped graphene quantum dots for cellular and deep-tissue imaging, Nano Lett. 13 (2013) 2436–2441.
[28] L. Qu, Y. Liu, J.B. Baek, L. Dai, Nitrogen-doped graphene as efficient metal-free electrocatalyst for oxygen reduction in fuel cells, ACS Nano 4 (2010) 1321–1326.
[29] X. Wang, X.H. Lou, Y. Wang, Q.C. Guo, Z. Fang, X.H. Zhong, H.J. Mao, Q.H. Jin, L. Wu, H. Zhao, J.L. Zhao, QDs-DNA nanosensor for the detection of hepatitis B virus DNA and the single-base mutants, Biosens. Bioelectron. 25 (2010) 1934–1940.
[30] L. Zhang, D. Peng, R.P. Liang, J.D. Qiu, Nitrogen-doped fraphene quantum dots as a new catalyst accelerating the coordination reaction between Cadmium(II) and 5,10,15,20-Tetrakis(1-methyl-4-pyridinio)porphyrin for Cadmium(II) sensing, Anal. Chem. 87 (2015) 10894–10901.
[31] J. Ju, W. Chen, Synthesis of highly fluorescent nitrogen-doped graphene quantum dots for sensitive, label-free detection of Fe (III) in aqueous media, Biosens. Bioelectron. 58 (2014) 219–225.
[32] T.V. Tam, N.B. Trung, H.R. Kim, J.S. Chung, W.M. Choi, One-pot synthesis of N-doped graphene quantum dots as a fluorescent sensing platform for $Fe^{3+}$ ions detection, Sens. Actuators, B 202 (2014) 568–573.
[33] A. Jinnarak, S. Teerasong, A novel colorimetric method for detection of gamma-aminobutyric acidbased on silver nanoparticles, Sens. Actuators, B 229 (2016) 315–320.
[34] H. Li, Z.Y. Sun, W.Y. Zhong, N. Hao, D.K. Xu, H.Y. Chen, Ultrasensitive electrochemical detection for dNA arrays based on silver nanoparticle aggregates, Anal. Chem. 82 (2010) 5477–5483.
[35] T. Takeshima, Y. Tada, N. Sakaguchi, F. Watari, B. Fugetsu, DNA/Ag nanoparticles as antibacterial agents against gram-negative bacteria, Nanomaterials 5 (2015) 284–297.
[36] Y.Y. Yin, Q. Liu, D. Jiang, X.J. Du, J. Qian, H.P. Mao, K. Wang, Atmospheric pressure synthesis of nitrogen doped graphene quantum dots for fabrication of BiOBr nanohybrids with enhanced visible-light photoactivity and photostability, Carbon 96 (2016) 1157–1165.
[37] C.H. Liu, Z.P. Li, B.A. Du, X.R. Duan, Y.C. Wang, Silver nanoparticle-based ultrasensitive chemiluminescent detection of DNA hybridization and single-nucleotide polymorphisms, Anal. Chem. 78 (2006) 3738–3744.
[38] İ. Karamollaoğlu, H.A. Öktem, M. Mutlu, QCM-based DNA biosensor for detection of genetically modified organisms (GMOs), Biochem. Eng. J. 44 (2009) 142–150.
[39] Z. Li, Y.N. Ni, S. Kokot, A new fluorescent nitrogen-doped carbon dot system modified by the fluorophore-labeled ssDNA for the analysis of 6-mercaptopurine and Hg (II), Biosens. Bioelectron. 74 (2015) 91–97.
[40] W.W. Zhao, P.P. Yu, Y. Shan, J. Wang, J.J. Xu, H.Y. Chen, Exciton-plasmon Interactions between CdS quantum dots and Ag nanoparticles in photoelectrochemical system and its biosensing application, Anal. Chem. 84 (2012) 5892–5897.
[41] J. Ju, W. Chen, In situ growth of surfactant-free gold nanoparticles on nitrogen doped graphene quantum dots for electrochemical detection of hydrogen peroxide in biological environments, Anal. Chem. 87 (2015) 1903–1910.
[42] X.H. Zhu, X.X. Zuo, R.P. Hu, X. Xiao, Y. Liang, J.M. Nan, Hydrothermal synthesis of two photoluminescent nitrogen-doped graphene quantum dots emitted green and khaki luminescence, Mater. Chem. Phys. 147 (2014) 963–967.
[43] S. Liu, J. Tian, L. Wang, Y. Zhang, X. Qin, Y. Luo, A.M. Asiri, A.O. Al-Youbi, X. Sun, Hydrothermal Treatment of Grass: a low-cost, green route to nitrogen-doped, carbon-rich, photoluminescent polymer nanodots as an effective fluorescent sensing platform for label-free detection of Cu(II) Ions, Adv. Mater. 24 (2012) 2037–2041.
[44] Y. Wang, Y.Y. Shao, D.W. Matson, J.H. Li, Y.H. Lin, Nitrogen-doped graphene and its application in electrochemical biosensing, ACS Nano 4 (2010) 1790–1798.
[45] F. Gao, P. Cui, X.X. Chen, Q.Q. Ye, M.G. Li, L. Wang, A DNA hybridization detection based on fluorescence resonance energy transfer between dye-doped core-shell silica nanoparticles and gold nanoparticles, Analyst 136 (2011) 3973–3980.
[46] H.W. Gao, M. Sun, C. Lin, S.B. Wang, Electrochemical DNA biosensor based on graphene and $TiO_2$ nanorods composite film for the detection of transgenic soybean gene sequence of MON89788, Electroanalysis 24 (2012) 2283–2290.
[47] K. Chen, H.Y. Han, Z.H. Luo, Y.J. Wang, X.P. Wang, A practicable detection system for genetically modified rice by SERS-barcoded nanosensors, Biosens, Bioelectron 34 (2012) 118–124.
[48] F. Li, J. Chao, Z.H. Li, S. Xing, S. Su, X.X. Li, S.P. Song, X.L. Zuo, C.H. Fan, B. Liu, W. Huang, L.H. Wang, L.H. Wang, Graphene oxide-assisted nucleic acids assays using conjugated polyelectrolytes-based fluorescent signal transduction, Anal. Chem. 87 (2015) 3877–3883.
[49] W. Wang, L. Zhan, Y.Q. Du, F. Leng, Y. Chang, M.X. Gao, C.Z. Huang, Label-free DNA detection on the basis of fluorescence resonance energy transfer from oligonucleotide-templated silver nanoclusters to multi-walled carbon nanotubes, Anal. Methods 5 (2013) 5555–5559.
[50] Z.S. Qian, X.Y. Shan, L.J. Chai, J.J. Ma, J.R. Chen, H. Feng, DNA nanosensor based on biocompatible graphene quantum dots and carbon nanotubes, Biosens. Bioelectron. 60 (2014) 64–70.
[51] T. Qiu, B. Zhang, Z.Y. Hu, J.H. Tang, H.P. Xie, B.R. Gu, Detection of DNA based on fluorescence resonance energy transfer of polyelectrolyte-protected CdTe quantum dots as energy donors, Analyst 137 (2012) 2608–2613.
[52] S. Huang, H.N. Qiu, C.S. Huang, W. Su, B.Q. Hu, A simple QD–FRET bioprobe for sensitive and specific detection of hepatitis B virus DNA, J. Fluoresc. 23 (2013) 1089–1098.

Please cite this article in press as: Y. Li, et al., A homogeneous assay for highly sensitive detection of CaMV35S promoter in transgenic soybean by förster resonance energy transfer between nitrogen-doped graphene quantum dots and Ag nanoparticles, Analytica Chimica Acta (2016), http://dx.doi.org/10.1016/j.aca.2016.10.031