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Determination of nitrogen deficiency-related microRNAs in plants using fluorescence quenching of graphene oxide nanosheets

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ABSTRACT

In this study, a microRNA (miRNA) biosensor based on fluorescence quenching technique is designed and developed to determine the miRNAs with altered expression during nitrogen deficiency in plants. The method is based on the difference between the fluorescence absorption of graphene oxide (GO) nanosheets in the present of gold nanoparticles-labelled DNA probe and that of GO after the DNA hybridization with target miRNA. Results revealed that the biosensor was capable of determining the concentration of 17 miRNAs related to the nitrogen stress in plants with acceptable specificity, limit of detection (LoD) and linear range. LoD values for the determination of studied miRNAs ranged from 45 to 420 aM. Furthermore, wide linear ranges were obtained for the determination of miRNA-156 (500-100,000 aM), miRNA-167 (500-20,000 aM), miRNA-171 (200-80,000 aM), and miRNA-398 (300-120,000 aM). The introduced biosensor can be a more reliable alternative to polymerase chain reaction (PCR) and northern blot for plant scientists who work on plant stress-related miRNAs.

1. Introduction

Earth is a plant-oriented planet and plants are studied at many levels from micro (molecular and cellular levels) to macro (morphological levels). Several sustainable methods have been developed to investigate the undesirable effects of biotic and abiotic stresses on plants at molecular levels [1]. Studies have shown that the altered expression of plant microRNAs (miRNAs) and their target genes during nutrient deficiency is a reliable method to investigate the plant behavior towards biotic and abiotic stresses [2]. miRNAs are endogenous, single-stranded noncoding RNAs which regulate the expression of multiple genes by degrading or blocking target mRNAs translation [3,4]. Hundreds of miRNAs are now characterized for their role in improving the stress tolerance mechanism of plants at the post-transcriptional level [5,6]. Due to the fact that nitrogen is an essential nutrient for plant growth, development and reproduction, and its deficiency is probably the most common nutritional problem affecting plants worldwide [7,8], the miRNAs involving the plant nitrogen stress and their target genes are deeply investigated by the researchers [9].

Today, several methods including northern blot [10], microarrays, and polymerase chain reaction (PCR) [11] are widely used for miRNA expression determination in biological samples. However, in general, these techniques have their own limitations: weak analytical characteristics [12], being time-consuming, and requiring sample pretreatment and complex laboratory experiments [13,14]. Instead, some methods such as nanoparticle aggregation, microfluidic methods, electrochemical techniques, and fluorescence quenching have been introduced as reliable analytical solutions for sensitive and selective determination of miRNAs [15–17]. Among these methods, fluorescence quenching technique [18], as a cost-effective method, is a simple way for the determination of miRNA concentration without the requirements to professional operations.

The application of graphene oxide (GO) nanosheets in molecular-level sensors has been increased in the last two decades thanks to the single-layer carbon atoms and carboxyl and hydroxyl functional groups in their two-dimensional honeycomb lattice [19,20]. GO nanosheets have been widely used to determine DNA hybridization based on the difference between the fluorescence absorption of GO in the presence of fluorophore-labelled single-stranded DNA probe and that of GO after the DNA hybridization with target microRNA [18].

Since fluorescence quenching is a rather simple and inexpensive technique, an investigation on its performance in the specific and sensitive determination of the most important miRNAs involving the plant nutrient stress is of interest. This is because, in this situation, plant scientists can simply develop this method to extend their work on the biochemistry of plant response towards nutrient deficiency. Therefore, the objectives of this study were: (a) to design and develop a GO nanosheets-based miRNA biosensor using fluorescence quenching

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technique, and (b) to evaluate the analytical performance of the biosensor in the concentration determination of miRNAs involving the nitrogen deficiency in plants.

2. Material and methods

2.1. Gold nanoparticle (Au NP) and DNA-Au NP preparation

The 12 nm Au NPs were prepared according to the method introduced by Turkevich et al. [21]. In brief, 90 mL of 1 mM chloroauric acid ($HAuCl_4$) was added to 50 mL of deionization water. In the next step, 1 mL of 1% trisodium citrate ($Na_3C_6H_5O_7$) was added when the boiling of the solution started. The produced mixture was then stirred and heated until its color changed. Finally, it was cooled down under stirring and stored at room temperature for further use.

The HPLC purified oligonucleotide sequences listed in Table S1 in Supporting information, validated by miRbase database, two random three-base mismatched sequence for each miRNA, and thiolated DNA probes listed in Table S2 in Supporting information were prepared at Private Laboratory of Biosensor Applications, Hamadan, Iran. The thiolated DNA probes were functionalized with Au NPs according to the method introduced by Zarei Ghobadi et al. [18]. Briefly, 500 mL of Au NPs was mixed with 1 mM thiolated DNA for 12 h. The DNA probe-Au NP solution was then centrifuged at 10,000 rpm to remove excess amounts of Au NPs and unconjugated DNA. The centrifuge continued for 15 min. Finally, the precipitate was dispersed in a 0.01 M phosphate (PO_4^{3-}) buffer at neutral pH, then cooled down to 4 $^{\circ}$C.

2.2. Optical sensing of target miRNAs

In order to detect target miRNA, the method of Zarei Ghobadi et al. [18] was used with slight modifications. Briefly, 10 mL of 100 mg/mL GO nanosheet prepared at Private Laboratory of Biosensor Applications, Hamadan, Iran, 5 mL of each DNA probe-Au NPs in 10 mM phosphate buffer (pH 7.0), 5 mL of 0.1 M salt (NaCl), and 5 mL of 5 mM magnesium chloride ($MgCl_2$) were incubated for 20 min. The target miRNA was then added and the mixture was heated to 90 $^{\circ}$C for 5 min and incubated for 30 min. Finally, the fluorescence intensities were measured before and after adding the target miRNA. UV-Vis absorption spectra of the mixture were recorded using a multi-mode microplate reader (SpectraMax iD5, Molecular Devices, USA). The biosensor response was measured using Eq. (1).

$$R = \frac{FI_R - FI_Q}{FI_Q} \quad (1)$$

where R is the biosensor response, FI_Q is the quenched fluorescence intensity after adding the Au NP-labelled DNA probes, and FI_R is the recovered fluorescence intensity after target miRNA hybridizing with DNA probe.

3. Results and discussion

3.1. Sensing mechanism for miRNA concentration determination

Similar to protein-coding genes, miRNA expression is upregulated or down-regulated in response to biotic and/or abiotic stress in plants. A search in bibliographic databases show that, in general, the expression of 17 miRNA sequences is altered due to the nitrogen deficiency in plants [2]. These miRNAs and their sequences in *Arabidopsis thaliana* are brought in Table S1 in Supporting information. They are small molecules with 21 nucleotides in their length (except miRNA-156 consisting of 20 nucleotides) which makes their determination difficult and challenging. The concept of proposed biosensor for the concentration determination of these miRNAs in plant extract is depicted in Fig. 1.

The photoluminescence property of GO nanosheet belongs to the recombination of localized electron-hole pairs within the domain of sp^2 carbon embedded in a sp^3 matrix [22]. The excitation of GO at 400 nm causes the emission fluorescence peak around 460 nm. For the bare GO, the fluorescence intensity at 460 nm is maximum (Fig. 1a). Since the surface of the GO nanosheet is highly heterogeneous and absorbs deoxyribonucleic acid, by adding gold nanoparticles (Au NPs) coated with DNA probes, fluorescence energy transfer occurs between Au NPs and GO which results in a decrease in fluorescence emission (Fig. 1b). The sequences of DNA probes are shown in Table S2 in Supporting information. Because of their high extinction coefficient and strong distance- and size-dependent optical properties, metallic nanoparticles, especially Au NPs have been widely used in optical biosensors [23,24].

In the presence of target miRNA, the probes which were absorbed on the surface of the nanosheet are desorbed from the GO surface and hybridized with the target. As a result, a large amount of the lost fluorescence emission of GO nanosheets is recovered (Fig. 1c). Therefore, the biosensor works on a simple basis: the higher the percentage of recovered fluorescence intensity, the higher the concentration of target miRNA in the studied sample. This simplicity is of very interest, especially when the biosensor is capable of determining several miRNAs with acceptable analytical performance without any requirements to apply changes in the protocol and/or the amount of reagents for each miRNA measurement.

3.2. Analytical performance of the biosensor

In order to achieve the analytical characteristics of the method in target miRNA determination including the linear range and limit of detection (LoD), aliquots of miRNA solution were added to inert electrolyte solution to obtain miRNA standards with concentrations in the range of 0–10^6 aM. Table 1 reveals the linear range and limit of detection (LoD) of the biosensor in the measurement of studied miRNAs. As can be seen, in general, the linear range is between hundreds of atto molars and dozens of femto molars. The linear range in this study was determined using a fitted linear line to experimental data with an R^2 more than 0.95. Several methods have been introduced to determine the LoD of an analytical method. In this study signal-to-noise ratio method [25] was used where this ratio was considered 3:1. Table 1 shows that the LoD values for the determination of studied miRNAs ranged from 45 to 420 aM.

According to Table 1, the linear ranges of the miRNA detection were not similar. Considering the fact that all the experiments were carried out with four replications, I think that the reason is the different hybridization intensities and the desorption amount of miRNAs from the surface of GO nanosheets. According to the table, the biosensor was capable of determining the concentration of miRNA-156, miRNA-167, miRNA-171, and miRNA-398 in a wide range. It has been shown that these miRNAs are involved in the plant response towards many abiotic stresses. For example, miRNA-156, miRNA-167, and miRNA-171 are down-regulated towards salt, drought, temperature, and UV B whilst miRNA-398 is up-regulated towards salt, temperature, and abscisic acid [9,26].

The main target genes and corresponding proteins which their regulation is altered by a change in the expression of the studied miRNAs are auxin response factor (ARF), sporocyteless (SPL), scarecrow-like 3 (SCL), Na^+/H antiporter (NHD), alkenyl hydroxalkyl producing 2 (AOP2), heme activator protein 2 (HAP2), apetala 2 (AP2), ATP sulfurylase (APS), sulfate transporter (SULTR), copper/zinc superoxide dismutase (CSD), cytochrome C oxidase subunit 5b-1 (cox5b-1), cytochrome C biogenesis protein (CCS1), ubiquitin-conjugating enzyme E2/phosphate 2 (UBC24/PHO2), nitrogen limitation adaptation (NLA), plantacyanin (ARPN). Plant response to these genes involves root development, signal transduction, shoot development, delayed vegetative phase change, Na^+ export, glucosinolate synthesis, nitrogen and copper homeostasis, floral development, and sulfur metabolism

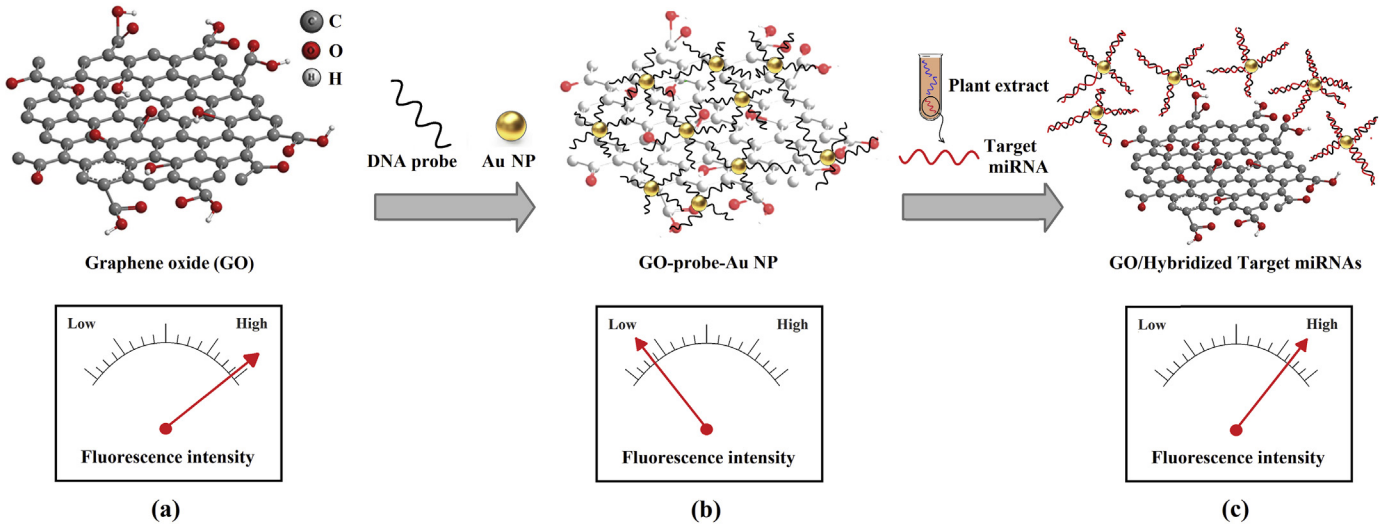

Fig. 1. Schematics of miRNA determination using an optical biosensor based on fluorescence quenching technique, (a) the bare GO shows the highest fluorescence intensity, (b) adding Au NP-labelled DNA probes quenches the fluorescence intensity, (c) target miRNAs hybridizing with DNA probes recovers the fluorescence intensity which the recovery amount is related to the miRNA concentration.

Table 1
Linear range and limit of detection (LoD) of the fluorescence quenching-based biosensor for the determination of nitrogen deficiency-involved miRNAs.

miRNA	Linear range (aM)			Limit of detection (LoD) (aM)
	Lower limit	Upper limit	R^2	
miRNA-156	500	100,000	0.97	200
miRNA-160	400	10,000	0.96	250
miRNA-167	500	200,000	0.98	85
miRNA-169	800	50,000	0.95	300
miRNA-171	200	80,000	0.96	150
miRNA-172	150	10,000	0.97	70
miRNA-395	500	60,000	0.95	420
miRNA-397	250	25,000	0.95	250
miRNA-398	300	120,000	0.97	80
miRNA-399	500	10,000	0.98	360
miRNA-408	300	30,000	0.95	180
miRNA-780	200	25,000	0.96	200
miRNA-826	150	15,000	0.96	100
miRNA-827	400	20,000	0.97	250
miRNA-842	150	10,000	0.95	100
miRNA-846	500	20,000	0.95	120
miRNA-857	400	10,000	0.96	45

[2].

According to Table 1, a wide range of the concentration of all the studied miRNAs can be measured by the proposed sensor. This shows that a biosensor based on the fluorescence quenching technique can be reliably used by plant scientists who perform accurate experiments on the effects of nitrogen deficiency on plant biochemical characteristics. Literature shows that plant researchers generally use qRT-PCR, northern blot, and microarrays to study the effects of plant stress on the expression of miRNAs and their corresponding target genes and proteins. These analytical methods show their up- and down-regulation whilst the intensity of the expression mostly remains hidden from the focus of the researchers. However, using a biosensor can simply and reliably determine the concentration of the miRNA in atto molars range.

Although biosensors have not yet become common methods for plant miRNA determination, electrochemical and optical biosensors are now widely used for the determination of human disease and cancer-related miRNAs [14]. Optical biosensors for the determination of miRNA-21 (a biomarker for early cancer diagnosis) [27], miRNA-155 (related to the human breast cancer) [28], and miRNA-196 (related to the pancreatic cancer) [29] are now introduced which can determine the target at atto molars. Furthermore, I recently developed an optical biosensor based on the nanoparticle aggregation for the determination of miRNA-1886 as a specific and selective indicator of drought stress in tomato [16]. However, the method introduced in the present study is very simple and can determine all the 17 miRNAs involving the nitrogen stress.

To study the specificity of the biosensor, firstly, 1000 aM of the target miRNAs were analyzed using the biosensor and the biosensor response which was the fluorescence intensity was recorded for the samples. Then, similar concentration of their corresponding two three-base mismatched miRNAs were analyzed and fluorescence intensities were recorded. All the measurements were carried out with five replications. Results in the form of mean and standard deviation values for each of the miRNAs are shown in Table 2. According to the table, in general., the fluorescence responses of the biosensor for the target miRNAs are six times higher than that for the mismatched miRNAs which indicates the acceptable specificity of the developed biosensor

Table 2
Biosensor response for 1000 aM of the studied miRNAs and two random three-base mismatched miRNAs (n = 5).

miRNA	Normalized fluorescence intensity					
	Main miRNA		Mismatched miRNA #1		Mismatched miRNA #2	
	Mean	SD	Mean	SD	Mean	SD
miRNA-156	0.96	0.03	0.10	0.04	0.14	0.04
miRNA-160	0.98	0.02	0.12	0.02	0.09	0.03
miRNA-167	0.82	0.04	0.11	0.03	0.12	0.04
miRNA-169	0.98	0.07	0.12	0.01	0.08	0.05
miRNA-171	0.92	0.07	0.15	0.03	0.13	0.01
miRNA-172	0.81	0.04	0.17	0.05	0.14	0.02
miRNA-395	0.85	0.05	0.19	0.01	0.14	0.04
miRNA-397	0.90	0.03	0.14	0.03	0.15	0.05
miRNA-398	1.00	0.02	0.13	0.05	0.17	0.03
miRNA-399	0.99	0.08	0.12	0.02	0.11	0.02
miRNA-408	0.83	0.07	0.15	0.02	0.15	0.03
miRNA-780	0.93	0.04	0.18	0.04	0.14	0.04
miRNA-826	0.99	0.05	0.16	0.03	0.12	0.05
miRNA-827	0.89	0.02	0.10	0.05	0.17	0.06
miRNA-842	0.96	0.03	0.17	0.04	0.12	0.04
miRNA-846	0.82	0.04	0.10	0.02	0.15	0.05
miRNA-857	0.88	0.05	0.13	0.01	0.16	0.02

towards the studied miRNAs. This specific behavior reveals the favorable hybridization of the DNA probe and target miRNA and the fluorescence recovery relative to the concentration of target miRNA.

4. Conclusions and future perspectives

Results show that the biosensor developed in this study can be a solution for reliable determination of miRNA instead of conventional analytical methods. It should be noted that electrochemical biosensors including amperometric [30] and impedimetric [31] are also developed for miRNA concentration determination, but the method used in this study is simple and cost-effective for plant scientists. It seems that higher concentration of miRNAs in the range of pico molars cannot be linearly detected by the introduced biosensor with R^2 values higher than 0.95. further studies on the analytical performance of this method should be carried out for the investigation of the performance of the biosensor for the determination of miRNAs with high amounts of concentration in plants. Moreover, machine learning can be used to develop intelligent biosensors [32] for the analyte determination in sensor non-linear ranges.

CRediT authorship contribution statement

Keyvan Asefpour Vakilian: Writing - original draft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2020.101576.

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