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RESEARCH PAPER

Decrease of catalytic efficiency of Photinus pyralis firefly luciferase in the presence of graphene quantum dots

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ABSTRACT

Objective(s): Firefly luciferase is a monooxygenase enzyme that emits flash of light during the enzymatic reaction. Luciferase has been used in many bioanalytical fields from ATP detection methods to in vivo imaging. In recent decades, focus has been carried out on nanoparticles for their fluorescence properties. Semiconductor quantum dots have unique tunable properties that turn them promising tools in biological and biomedical researches, as nanosensors, photo-electrochemical and light-emitting devices. Carbon-based nanoparticles such as graphene quantum dots (GQDs) have useful benefits such as low toxicity, suitable luminescence and easy preparation.

Materials and Methods: In this study, recombinant P. pyralis luciferase was expressed and purified based on N-terminal His-tag and then kinetic parameters of enzyme activity such as Km and Vmax values in presence and absence of GQDs were calculated.

Results: The results showed that Km for ATP and luciferin substrates in the presence of GQDs were increased. Fluorescence spectroscopy showed significant changes in protein structure or in fluorescence spectra and decrease in the activity of the luciferase in presence of GQD. Both loss of activity and increase of substrates Km showed decrease of catalytic efficiency presumably through structural alteration.

Conclusion: From these data it can be concluded that the protein structure under the influence of GQD may have changed that lead to alteration of enzyme activity.

Keywords: *Bioluminescence, Graphene, Luciferase, Quantum dot*

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INTRODUCTION

In all of light emitting organisms, process of light emission in a living species is performed by a type of chemical reaction called bioluminescence [1-4]. Firefly luciferase (EC 1.13.12.7) is a 62 KDa protein, which has been very useful in biological research field for various purposes such as monitoring transcriptional activities [5-7], quantification of ATP, analyzing and assessing of gene expression and also as a tool in bioluminescence imaging [6, 8, 9]. Luciferase-based assays as a reporter gene assay are sensitive, measureable, fast, reproducible, and achievable [6, 10, 11]. The firefly luciferase catalyzes a chemical reaction with two steps: adenylation of D-luciferin by ATP in presence of Mg^{2+} to produce luciferyl adenylate and pyrophosphate which is followed by oxidation of luciferyl adenylate by molecular oxygen to towards dioxetanone [12-14], an excited state of oxyluciferin, with in[ter]conversion between the two keto and enol forms [13]. Light emission is a consequence of oxyluciferin return to the ground state [14-17] at room temperature and pH 7.8, with an emission spectrum with a maximum wavelength at 562 nm (yellow-green) [8, 18, 19]. Quantum dots as a new photostable semiconductor nanocrystals with the typical dimensions ranging from 2-10 nanometers have roughly 200 to 10,000 atoms which are relatively comparable to a large protein in their sizes [20, 21]. They have wide excitation spectra and narrow wavelength with capability for conjugation to biological targets, such as proteins, antibodies and

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nucleic acids [22-24]. Graphene quantum dots (GQDs) as a novel nanomaterial have been used in various fields because of their unique properties among these nanoparticle [25, 26] .GQDs caught great interest due to its significant properties such as, low cell toxicity, biological compatibility, high image and easily preparation [27-31].

It is important to see what the behavior of nanoparticles like QDs can affect on crucial enzymes such as luciferase. Some of these behaviors can cause the enzyme stable or even no dramatic changes can be seen. Hence, in this study, due to role of quantum dots in protein thermostability for the first time the kinetic properties of firefly luciferase from *P. pyralis*, in the presence and absence of GQDs were measured. Then the structural changes of the enzyme were studied by intrinsic fluorescence spectroscopy.

Materials and methods

Materials

D-luciferin (Resem, The nethelands), $MgSO_4$, ampicillin, lactose (Sigma), and Ni-NTA-Sepharose affinity column were purchased from Novagen (Germany) [1].

Preparation of GQDs

The GQDs were produced from graphene sheets (GSs) through a hydrothermal process [32]. To prepare the water-soluble GQDs (9-15 nm), 270 mg of GO was dissolved in 20 ml of DMF and sonicated for 30 min as a mixture (120W, 100kHz) and then transferred to a poly (tetrafuoroethylene) (Tefon)-lined autoclave (30 ml) and heated at 200 °C for 5 h which is followed by cooling to RT, after completion of the reaction. Yellow-brown supernatant with black precipitates were filtered twice and a yellow brown suspension was collected. Finally, obtained GQDs were dried using rotary evaporator, and subsequently dissolved in water.

Expression and purification of luciferase enzyme

For expression of enzyme, 10 ml of LB (Luria-Bertani) Liquid medium containing 50 µg/ml ampicillin was inoculated with a fresh bacterial colony harboring the pET-16b expression plasmid and cultured at 37 °C overnight. Then 250 ml of fresh medium was inoculated with 500 µl overnight culture and incubated at 37 °C with shaking (150 rpm) until the OD reached 0.6–0.9 at 600 nm. Then, lactose as an inducer (8 mM) was added to the medium and incubated at 22 °C overnight with the same condition previously mentioned. After that, centrifugation at 5000 g for 15 min was performed to precipitate the cells and then the cell pellet was resuspended in lysis buffer [50 mM Tris, 300 mM NaCl, 10 mM imidazole, (pH 7.8)]. Purification of His6-tagged (polyhistidine-tag) fusion protein was performed by Ni-NTA (nickel-Nitrilotriacetic acid) affinity chromatography column and finally protein concentration was determined by Bradford assay and using bovine serum albumin as standard [34]. After addition of luciferin and Mg+2 and ATP to the purified firefly luciferase, the color of the emitted light of luciferase reaction was observed.

Luciferase activity measurements

Luciferase activity was measured by using Berthold Luminometer FB 14. Enzymatic reactions were carried out by the injection of 5 µL standard solution (containing Luciferin (2 mM), $MgSO_4$ (10 mM), ATP (4 mM), and Tris buffer (50 mM)) and 5 µl enzyme (0.6 mg/ml) in the Luminometer.

Luciferase activity assay in presence and absence of GQD

Enzymatic reactions were carried out by the injection of 5 µl standard solution and 2.5 µl enzyme (0.6 mg/ml). Then the solution was mixed with 2.5 µl of various concentrations of water soluble GQD (650, 320, 160, 80, 40, 20, 10 µM) in the Luminometer.

Characterization of kinetic parameters in presence and absence of GQD

Kinetic parameters such as Km and Vmax were calculated by using PRISM software. To determine luciferin Km, 12 µl of assay reagent including 10 mM $MgSO_4$ and 4 mM ATP in 50 mM Tris (pH 7.8) were mixed with 8 µl of different concentrations of luciferin (0.004–5 mM) in a tube. The reaction initiation was happened by adding 5 µl of enzyme in the presence and absence of GQD to 5 µl of substrate, and then light emission was recorded over 10 s. The kinetic parameters of ATP was performed under the same condition. 2 µl from various concentrations of ATP (0.001 to 4 mM) were mixed with 16 µl of assay reagent, containing 10 mM $MgSO_4$ and 2 mM luciferin in 50 mM Tris (pH 7.8). The reaction was initiated by adding 2 µl of enzyme in the presence and absence of GQD (80 µM), and then light emission was recorded over 10 s.

Thermal stability and remaining activity of luciferase enzyme in the presence and absence of GQD

The purified luciferases (0.6 mg/ml) in the presence of GQD with density of 80 µM were incubated in the range of 20–45°C for 5 min, to study thermal stability. Enzyme activity was measured at room temperature (25°C). The remaining activity of the luciferase enzyme in the presence and absence of GQD was investigated. For the measurement of remaining activity, the purified enzyme and enzyme in the presence of GQD were incubated at 32°C for 50 min and after about 5 min, the remaining activity was recorded as a percentage of the original activity. An enzyme solution kept on ice was used as the control for enzyme activity(100%).

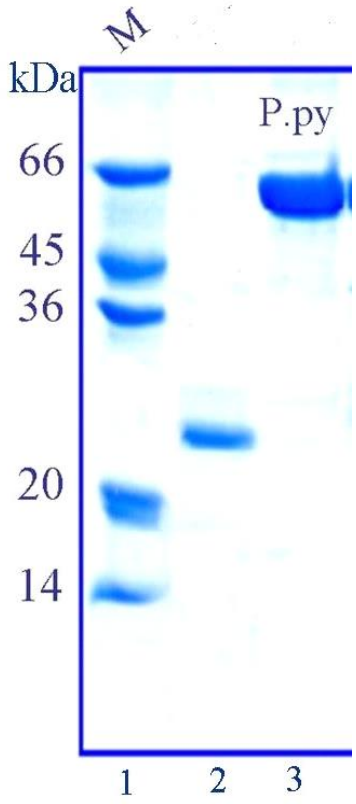

Fig1. The SDS-PAGE analysis of luciferase after column purification. M: Molecular weight marker; Lane 2: trypsin (as a control marker); Lane 3: eluted firefly luciferase (fraction 2)

Fluorescence measurements

Intrinsic fluorescence emission spectroscopy of enzyme in presence of different concentrations of GQD were measured by PerkinElmer LS55. The *P. pyralis* luciferase was excited at 295 nm and the emission spectra were recorded between 300 and 400 nm. All the fluorescence measurements carried out at room temperature in final volume of 60 µl. The excitation emission slit widths were set at 5 and 10 nm respectively and protein concentration was 0.6 mg/ml for each assay.

RESULTS

Firefly luciferase expression

The purification of expressed protein was performed based on the 6His-tagged fusion, by affinity (Ni-NTA_Sepharose) chromatography. Purification confirmation was carried out by SDS-PAGE analysis of eluted enzyme (Fig 1). (Moreover, after adding the substrate to the purified luciferase, we could see the emitted light with naked eyes in a dark room).

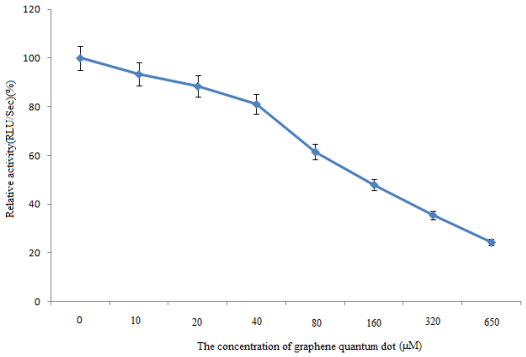

Fig2. Effect of different concentrations of graphene quantum dots on luciferase enzyme activity

Assay of luciferase in presence and absence of GQD

UV/Vis absorption spectroscopy of synthesized GQDs revealed an absorption shoulder in the visible region at 320 nm which is associated with n-π* transition and a strong absorbance at 230 nm attributed to π-π* transition, similar to previous reported studies for GQDs. Surface functional groups on GQDs showed specific characteristic corresponding to epoxide, C=O, and O-H by FTIR spectroscopy. Moreover, Raman spectrum of GQDs showed two main peaks: the D-peak at 1301 cm−1 and the G-peak at 1524 cm−1. The relative intensity of the D-peak to the G-peak (ID/IG) was 0.71. In the study of the effect of various concentrations of GQD (650, 320, 160, 80, 40, 20, 10 µM) on the activity of luciferase enzyme was studied and as shown in (Fig 2), decreasing the activity of enzyme was observed by increasing the GQD concentration.

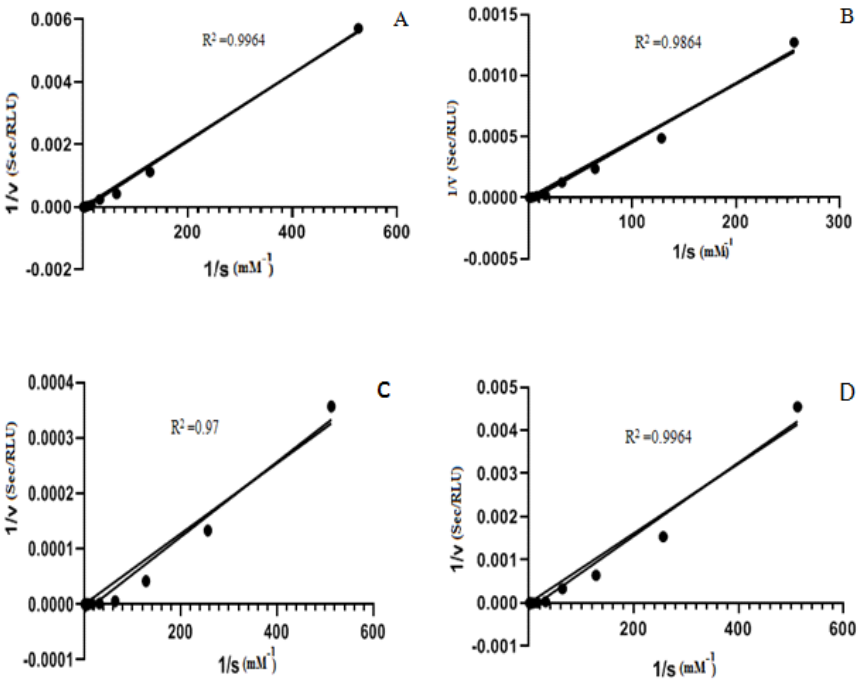

Fig3. Line-weaver Burk plot of firefly luciferase in the (A) absence and (B) presence of GQDs for ATP; and (C) absence (D) and presence of GQDs for luciferin

Kinetic properties

Kinetic properties of enzyme in presence and absence of GQD were studied after drawing line Weaver-Burk plot (Fig 3). Comparison of kinetic values for enzyme substrates in the presence and absence of GQD is presented in (Table 1).

Table1. Kinetic parameters of P. pyralis luciferase in presence and absence of GQD

	Km (μM) ATP	Km (μM) Luciferin	Vmax(RLU/mg/s) ATP	Vmax(RLU/mg/s) Luciferin	pH
Luciferase	125	70	1.25×10^{4}	1×10^{5}	7.8
Luciferase + GQD	166	80	3.33×10^{4}	1×10^{4}	7.8

The results showed that the Km of enzyme for both substrates in the presence of GQD was increased. Vmax for Luciferin was decreased and in the case of ATP was increased compared to free enzyme.

Thermal stability and remaining activity of luciferase in the presence of GQD

The thermal stability analysis of luciferase in the presence of GQD showed a decrease in thermostability for luciferase (Fig 4 A). The remaining activity analysis of luciferase in the presence of GQD during 50 minutes at 32°C showed also decrease in activity for luciferase (Fig 4 B).

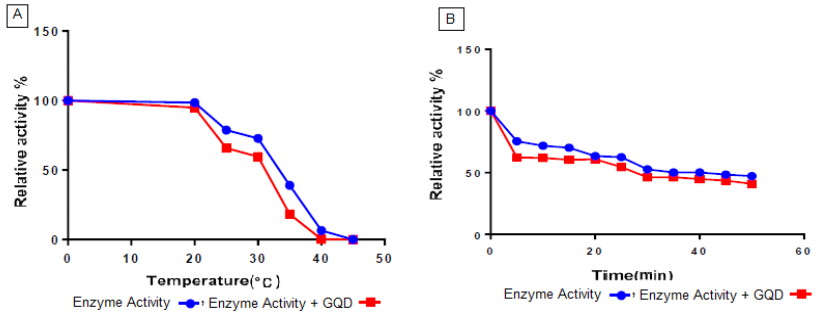

Fig4. (A) Comparison of thermal stability of luciferase in the presence and absence of GQDs (B) Comparison of the remaining activity of luciferase in the presence and absence of GQD

Intrinsic fluorescence changes of luciferase in presence of different concentration of GQD

Intrinsic fluorescence spectra of enzyme in presence of different concentrations of quantum dots was measured (Fig 5). The intrinsic fluorescence is caused mainly by tryptophan residues-convenient natural probes in firefly luciferase which has two tryptophan with excitation at 295 nm. As indicated in Fig 5, enzyme fluorescence intensity was decreased by increasing concentration of GQDs.

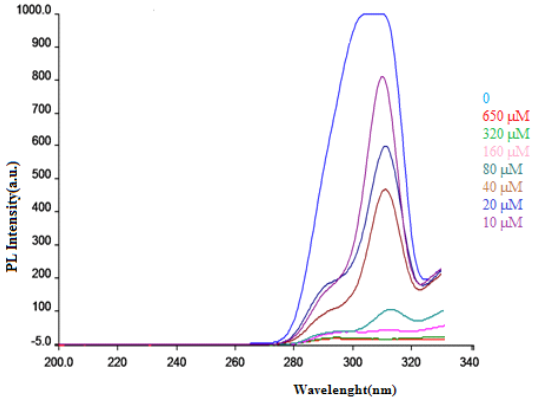

Fig5. Intrinsic fluorescence spectra of native luciferase enzyme and luciferase in the presence of GQD. Spectra were recorded at 25 ° C and pH 7.8. The concentration of proteins was 0. 6 mg/ml. The excitation wavelength was 295 nm

DISCUSSION

Many studies have focused on firefly luciferase for years and proven to have potential applications in many bioanalytical fields such as measuring microbial contaminations in the foods and medicine purposes [12, 25]. This enzyme catalyzes the light emitting reaction, under the term of Bioluminescence in organisms [12]. Recently, nanostructured materials such as gold nanoparticles, magnetic nanoparticles and quantum dots gained widespread attentions that made them as a suitable platform for enzyme modifications [35, 36]. However, there are some works referring the modification of luciferase by nanoparticles like GQDs to evaluate the enzyme performance. Some of these studies have reported increase or decrease of enzyme activity in reaction conditions. Stability of the luciferase was measured in the presence of Sucrose, Sorbitol and Proline at 35 °C which showed increase in enzyme stability [37]. Firefly luciferase was showed improved activity and stability in presence of different environments like ionic liquids [31, 32], hydrophobin [33], magnetic nanoparticles [34, 35], immobilization on Metal organic frameworks (MOFs) matrices [36] and nanoparticles [37]. Zhou Xiaoyan and coworkers studied the *Pseudomonas cepacia* lipase modification by graphene quantum dots. GQD-modified lipase showed a higher activity, stability and thermostability [28]. It was not observed any significant changes in the secondary structure of the enzyme after interaction of Citrate-coated silver nanoparticles (AgNPs) with firefly luciferase [38]. Due to the high efficiency and good stability of GQDs, Sanju Gupta and co-workers designed the glucose biosensor by immobilization of the glucose oxidase (GOx) on GQDs modified glassy carbon (GC) electrodes. They observed that GOx-GQDs biosensor responded efficiently and linearly to the presence of glucose from 10 µM to 3 mM [39]. Functionalized GQDs were used for immobilization of horse radish peroxidase (HRP) to detect hydrogen peroxide. This electro-chemical biosensor showed acceptable sensitivity values of 0.905 and 7.057 µA/mM and also a fast response time of ~2−3 s [40].

In order to find out what effects GQDs have on firefly luciferase, we evaluated the kinetic characteristics, thermal stability and remaining activity of luciferase in presence and absence of GQDs. According to the kinetic studies (Table 1), Km for luciferin and ATP as substrates of enzyme in the presence of GQD, increased. Also Vmax increased for ATP and decreased for luciferin. From these data it can be concluded that GQDs may reduce the enzyme tendency towards its substrates. Moreover, GQDs decreased enzyme thermal stability (Fig 4 A) and remaining activity of luciferase within 50 minutes at 32 °C in presence of GQD (Fig 4 B). Also, by increasing concentrations of GQDs, the activity and the emission spectrum of enzyme, continuously decreased (Fig 5). According to previous experiences, in all of the luciferase structural analysis loss of thermostability had been accompanied with structural perturbation which brought about with loss of intrinsic tryptophan fluorescence [41-43]. From these data it can be concluded that the protein structure under the influence of GQD may have changed that lead to alteration of enzyme activity.

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