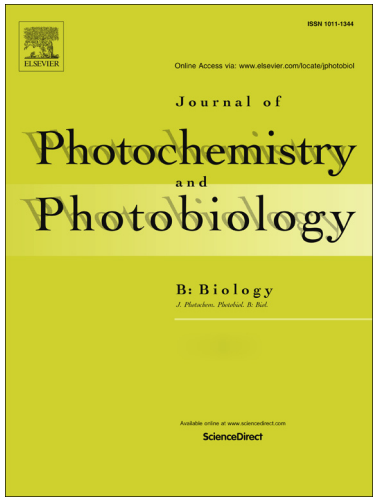

Chitosan-induced Au/Ag nanoalloy dispersed in IL and application in fabricating an ultrasensitive glucose biosensor based on Luminol-H_2O_2-Cu^{2+}/IL chemiluminescence system

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Chitosan-induced Au/Ag nanoalloy dispersed in IL and application in fabricating an ultrasensitive glucose biosensor based on Luminol-H_2O_2-Cu^{2+}/IL chemiluminescence system

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Abstract:

A novel glucose biosensor based on the Chemiluminescence (CL) detection of enzymatically generated hydrogen peroxide (H_2O_2) was constructed by one covalent immobilization of glucose oxidase (GOD) in glutaraldehyde-functionalized glass cell. In following, chitosan-induced Au/Ag nanoparticles dispersed in ion liquid (IL) were synthesised and immobilized on it. Herein, chitosan molecules acted as both the reducing and stabilizing agent for the preparation of NPs and also, as a coupling agent GOD and Au/Ag alloy NPs. In addition to catalyse luminol CL reaction, these NPs offered excellent catalytic activity toward hydrogen peroxide generation in enzymatic reaction between GOD and glucose. The used IL in fabrication of biosensor increased its stability. Also, IL alongside Cu^{2+} accelerated enzymatic and CL reaction kinetic, and decreased luminol CL reaction optimum pH to 7.5 which would enable sensitive and precision determination of glucose. Under optimum condition, linear response range of glucose was found to be 1.0×10^{-6}_ 7. 5 $\times 10^{-3}$ M, and detection limit was 4.0×10^{-7} M. The CL biosensor exhibited good storage stability, i.e. 90% of its initial response was retained after 2 months storage at pH 7.0. The present CL biosensor has been applied satisfactory to analysis of glucose in real serum and urine samples.

Keywords: Biosensor, Glucose, Chemiluminescence, Covalent immobilization, Au/Ag alloy NPs, Ion liquid, Cu^{2+}

1. Introduction

Biosensors have attracted much attention during recent times because of the potential applications of these devices in the clinical diagnostics, environmental monitoring, pharmaceuticals, and food processing industries due to their fast response and ease of operation [1, 2]. For enzyme-based biosensors, the biological recognition molecule is enzyme, which can catalyze many chemical reactions in living system. The free enzyme usually is not stable and cannot be easily reused. The development of enzyme immobilization techniques can strengthen the needs for enzyme stability and reuse. The immobilization processes make use of a soluble enzyme to attach onto an insoluble solid support [3].

The chemiluminescence-based biosensors are developing due to their high sensitivity and excellent performance. The selectivity, sensitivity and lifetime of the chemiluminescence (CL) biosensors would be greatly depending on the following points: (1) the careful selection of CL reagents responding to the definite species, (2) the way to immobilize the reagents, and (3) the substrates selected for reagents immobilization. Reagents immobilization onto proper substrates plays an important role in the development of the high-quality CL-based biosensors [4].

In this regard, the immobilization of enzyme is a key step for constructing an enzyme-based CL biosensor. It offers several advantages for chemical analysis such as convenient handling of enzyme preparation, enhanced stability, easy separation of the enzyme from the product, enzyme reusability [5], and reproducibility of the analytical method. The lifetime of an enzyme-based biosensor usually depends on how long the biological activity of the immobilized enzyme can be retained, and the enzymatic activity and stability depend on the immobilization strategy of enzymes. Numerous physical and chemical techniques of immobilization such as covalent linkage, physical adsorption, cross-linking, encapsulation, and entrapment have been used for stabilizing enzymes in the development of biosensors [5,

6]. But all the methods present advantages and drawbacks. The covalent linkage as Compared to other enzyme immobilization methods has many advantages, including effectively and durability, reproducibility, and lack of diffusion problems but varies the enzyme performance easily [7, 8]. Therefore, it is necessary to adjust this problem of biosensors based on covalent link with introduction and application an improver composition of enzyme activity.

Emergence and recent advance of nanoscience and nanotechnology open new opportunities for the application of nanoparticles in bioanalysis [9, 10]. This could be attributed to the unique chemical and physical properties of nanoparticles. In particular, gold and silver nanoparticles attract great scientific and technological interest because of easy preparation, good biocompatibility and relatively large surface-to-volume ratio [11].

The similar dimensions of enzyme and gold nanoparticle enable the interaction of enzyme and gold nanoparticle, and enzyme could easily absorbs on the surface of nanoparticle. While, in general, the consequent destructive changes of nanoparticle toxicity in the activity of proteins upon binding to nanoparticles can expect, there is a potential positive outcome; using of gold nanoparticles induce protein stability instead of enzyme degradation and increase the activity of enzymes via immobilization at surfaces, which are very useful for constructing enzyme-based biosensors [12, 13].

As mentioned above, gold nanoparticle is an efficient catalyst at enzymatic activity without degradation effects on enzyme [12, 13]. On the other, some of nanoparticles such as Ag and platinum exhibit the stronger CL catalytic activity than gold NP, although platinum NP is very expensive [14]. Due to all of the above information and nature of the good biocompatibility of Au and Ag NPs, it is guessed that use of Ag/Au alloy NPs for fabrication of sensitive CL biosensor for glucose analysis is very noteworthy.

To the best of our knowledge, heretofore nanoparticles just by using sol-gel method have been participated in the preparation of biosensor [13, 15, and 16]. To achieve this goal, the

stabilization of nanoparticles on the CL Cell surface along with covalent linked-enzyme for enhancing biosensor efficiency and stability; there is a need to introduce new initiative.

Chitosan (Cs) is a natural polysaccharide polymer with abundant reactive amino and hydroxyl functional groups [17] that can help us to build our ideal biosensor. it has several interesting properties: First, chitosan solution readily forms a stable and biocompatible film on the solid supports such as glassy carbon and glass [17- 19], it was reported to produce Au NPs by acting as stabilizer and reductant agent by virtue of its abundant amino and hydroxyl groups [20], and also chitosan has been widely used as the immobilization matrix because it could provide a suitable microenvironment similar to the native environment of the biomolecules to retain their bioactivity [21-23]. Hence, synthesis of Au/Ag chitosan-induced Au/Ag alloy NP and introduction of it at this work can have several advantageous: (1) chitosan on it provide covalently binding of nanoparticle along with the enzyme on the cell surface as enzyme stabilizer agent and enhancer of its activity, (2) removal of disadvantageous un-covalent methods, (3) chitosan ability on forming film increases biosensor stability against environmental interferences, (4) using of chitosan as the reducing and stabilizing agent in the synthesis of Au/Ag alloy NPs, and also as immobilization matrix of NPs on the surface of CL cell precludes applying additional materials in preparation of biosensor that can prevent possible interference of substances involved in the reaction, (5) the immobilized nanoparticles on the surface of the biosensor act as the enhancer of luminol CL reaction, (6) finally, possibility innovative design of highly sensitive and stable biosensor for qualitative analysis.

Despite the mentioned initiatives, There are still some problems in construction an ultrasensitive glucose biosensor; (1) the generation of enzymatically H_2O_2 has a relatively slow kinetic in comparison of chemically production reaction and it can lead to problem in CL analysis, (2) the alkaline optimum pH of luminol CL reaction.

Imidazolium ion based ion liquids (IL) have recently been shown as promising compounds for preparation and stabilization of nanomaterial, and have found application in transition metal catalyzed CL reactions [24-27]. Ion liquids with copper ion form an imidazolium ring/copper ion complex at neutral pH in presence of phosphate buffer. The optimum pH of luminol CL reaction could be softened through the use of the complex [28]. It also can improve the kinetic parameters of the enzyme in spite of unchanged catalytic efficiencies [28].

This paper describes the synthesis and characterization of chitosan-induced Au/Ag alloy NPs dispersed in IL, and use of them in the construction of a novel biosensor for determination of glucose in pharmaceuticals sample by CL reaction. Glucose oxidase (GOD) is inexpensive and practically applied in clinical and pharmaceutical analysis. So, it is chosen as a model enzyme system. Such fabrication of biosensor exhibited good accuracy, high sensitivity and long-term stability, and can easily adapted with other enzymes. The details of the preparation, Characterization, optimal conditions and possible application of biosensor were described as follows. Finally, the constructed CL biosensor was used to determine the glucose concentration in real serum and urine samples with the satisfactory results.

2. Materials and methods

2.1. Materials

Glutaraldehyde (25%), 1-Ethyl-3-Methylimidazolium ethylsulfate ([Emim][$EtSO_4$]) (99%) and chitosan (MW $1.9-3.1\times10^5$; 85%—90% deacetylation) were purchased from Aldrich. Sodium phosphate monobasic monohydrate (99%), disodium hydrogenophosphate (99%), Glucose, copper (II) dichloride (99%), $HAuCl_4\cdot4H_2O$ (48% w/w) and $AgNO_3$ were supplied from Merck chemical company. Glucose oxidase (GOD) solution (100U/ml) was

obtained from Molecular Probes (Zist chemistry, Tehran, Iran). The appropriate amount of luminol (Fluka) was dissolved in 0.10 M NaOH solution to obtain a 0.025 M stock standard solution. The stock solution of fluorescein isothiocyanate (FITC) (0.1 mg/ml) was prepared in phosphate buffer (0.1 M, pH=7.5) and protected from light. 0.1 M phosphate buffer solutions with various pH values were prepared by dissolving an appropriate amount of Na_2HPO_4 in water and adjusting the pH values with 0.1 M HCl or NaOH solutions. All of the other used reagents were analytical grade and supplied from sigma. Doubly deionized water was used throughout the study.

2.2. Apparatus

Chemiluminescence measurements were carried out by a Sirius tube luminometer (Berthold detection system, Germany) with a photomultiplier tube detector in a light-tight globular bottom glass cell of 10 mm diameter. A 3030 Jenway pH meter (Leeds, UK) was used for pH measurement. Transmission electron microscopy (TEM) images were recorded on a Philips CM10 transmission electron microscope. Absorption and fluorescence spectra were obtained using UV–Vis spectrophotometer (Cecil, CE5501) and LS-3B Perkin-Elmer spectrum instrument, respectively.

2.3. *Synthesis of chitosan-induced gold–silver alloy NPs dispersed in ion liquid*

The chitosan- induced Au/Ag alloy NPs dispersed in ion liquid were synthesised base on microwave method [20, 29] and our previous experience [30]. All glassware were thoroughly cleaned with freshly prepared aqua regia (HNO_3: HCl = 1:3, V/V) and rinsed extensively with doubly distilled water before use. In a typical experimental, 2.5ml of both $HAuCl_4$ and $AgNO_3$ (5mM), and 2.5 ml of [Emim][$EtSO_4$] (1.2 M) were mixed and sonicated at room temperature for 30 min in order to homogeneously distribute the metal ions in the ion liquid media. Then, the mixture were added to 1% acetic solution of CS (45 ml, 1 mg/ml) in a

250-ml round-bottom flask (pH=4–5). After stirring for 5 minutes, the flask was placed in a microwave oven and connected to a condenser. Subsequently, the reaction mixture was irradiated by microwave in a predesigned mode (200W- 30 min) yielding a brown-red colloidal solutions. Finally, the cooled solutions were stored at 4 °C in the refrigerator until use. The Au/Ag alloy NPs formation is confirmed by the UV–visible absorption spectra (Fig.1a) and TEM data (Fig.1b). Statistical analysis of TEM data revealed that the average diameters of Au/Ag alloy (3:2) nanoparticles [31] were 12.0 ± 2.0 nm.

< Fig. 1a & 1b>

2.4. Preparation of the glucose CL biosensor

The immobilization of glucose oxidase on CL glass cell consists of the following steps:

At first, the CL glass cells were treated with freshly prepared aqua regia (HNO_3: HCl = 1:3, V/V) to eliminate contaminations.

(1) After washing with deionized water, 2 ml hydrofluoric acid 40% (v/v) was added to the CL cell for 30 min at room temperature (RT) to ensure the complete removal of the silica cladding and the presence of active hydroxyl groups on the surface of the CL cell, then cell was washed twice with deionized water and dried under high vacuum.

(2) The CL cell were exposed to 2 ml phosphate buffer (PH=5, 0.1M) for 5 min, after remove buffer, the glutaraldehyde solution (5% W/W) as a cross-linking agent was added to cell for 6h. At the acidic condition, the glutaraldehyde solution reacts with active hydroxyl groups in the surface of CL cell in a good way.

(3) The cell was contacted with each of glucose oxidase and chitosan-induced Au/Ag alloy NPs dispersed in ion liquid for 5h, respectively.

(4) The cell was retained under vacuum overnight to CS form a film over CL cell that protects it.

(5) Finally, the enzyme-NPs-immobilized CL cell was rinsed with phosphate buffer (pH 7.5) twice alternately to remove the un-cross-linked and unabsorbed reagents. The enzyme-NPs-immobilized cell was stored at 4 ˚C until further use.

2.5. Glucose chemiluminescence assays

100 µL glucose of an appropriate concentration (in 0.1 M phosphate buffer pH 7.5) was transferred into the enzyme-NPs-immobilized cell. The cell was closed and the contents were stirred for 1 min; then stirring was stopped, 100 µL phosphate buffers (pH 7.5, 0.1 M) and 30 µl of 0.025 M copper ion (II) were added to it and then, the cell was inserted into a luminometer instrument. Glucose assay was easily carried out by the following method: 150 µL luminol (the optimized concentration) was injected into the immobilized CL cell for catalytic oxidation of glucose. Glucose concentration was quantified by the CL peak height. When reaction conditions were optimized, the CL relative intensity corresponded to the normalized maximum light intensity.

2.6. Glucose oxidase labelling with Fluorescein isothiocyanate

The bounding of enzyme to the active glass cell was confirmed using glucose oxidase labelled with fluorescein isothiocyanate. FITC was coupled to enzyme similar to Marshall labelling method [4]. 0.5 mg FITC was dissolved in 5 ml of phosphate buffer (pH=7.5, 0.1 M). After shaking it up to become a homogenized solution, 1 ml of FITC (0.1 mg/ml) solution was added slowly to the glucose oxidase solution (100U/ml) and the mixture was allowed to stand at 4 °C for 12 h. Then, 2 ml of the mixture was used in the preparation steps of biosensor instead of unlabelled enzyme. Unbounded FITC-labelled enzyme was separated

by twice rising with distilled water. The activity of the fluorescein isothiocyanate labelled glucose oxidase was found to be equal to that of the native enzyme within the experimental error [32].

2.7. *Confirm immobilization of glucose oxidase*

Direct studying of fluorescence of the labelled enzyme on the CL cell was not possible because of the high stray light. The immobilization of enzyme was proved by measuring the fluorescence of FITC released in the solution. The fluorescence spectra of the solution was obtained after treatment of the immobilized CL cell, bearing the FITC labelled glucose oxidase, with an aqueous solution containing 4% HF and 1% Triton X-100 for 20 min. This solution etches the CL cell surface releasing the labelled enzyme into the solution.

3. Results and discussion

In order to investigate the general effects of the immobilized reagents, biosensor performance was evaluated in the absence and presence of them. As expected, the strong CL signals were observed in the presence all of them. Also, no measurable CL signals were obtained in the absence each of them (Fig.2).

< Fig. 2>

Also, effect of Cu^{2+} on biosensor performance was investigated. As shown in Fig.3, the presence of Cu^{2+} increases the kinetic of biosensor response. It seem to be related to catalytic effect of $[Emim][EtSO_4]/Cu^{2+}$ complex formed in the presence of phosphate on kinetic of enzymatic reaction [28]. At following each of reagents were studied in detail:

< Fig. 3>

3.1. Effect of concentration of glutaraldehyde

Without glutaraldehyde, the CL intensity was very weak, unstable and declined rapidly. It is related to small amount of enzymes adsorbed physically on the CL cell in absence of glutaraldehyde and also, the immobilized enzymes easily dropped from the CL cell. Covalent bonding can be used to achieve the effective immobilization of enzymes to CL cell. Covalent methods are based on the linking reaction between the terminal functional groups of the protein and the reactive group on the solid surface of CL cell tthrough mediation a cross-linking agent. In this biosensor, glutaraldehyde was used as a cross-linking agent for enzyme immobilization, the hydroxyl group of the active glass cell and amine group of the enzymes can react with glutaraldehyde functional groups. The biosensor response biosensor was studied when the enzyme-immobilized CL cell was cross-linked by various concentrations of glutaraldehyde solution. The results (Fig. 4) showed that the CL intensity increase with increasing glutaraldehyde concentrations in the range of 0 to 5.0% (w/w). But higher glutaraldehyde concentrations (>5.0%) resulted in lower CL intensity because higher glutaraldehyde concentrations would denature most of the enzymes and lead to a decreasing in sensitivity and selectivity of the biosensor. As a result, 5.0% (w/w) glutaraldehyde solution was chosen as the optimal concentration of cross-linking agent.

< Fig. 4>

3.2. Effect of immobilization time

The duration of glutaraldehyde, Au-Ag alloy NP and enzyme immobilization on CL cell would effect on amount of each of the immobilized reagents on the CL cell, which is effective on the sensitivity of the biosensor. In regard glutaraldehyde, as shown in Fig. 5, it can be resulted that the CL intensity increases with increasing immobilization time from 2 to 6 h because the amount of reacted glutaraldehyde with active hydroxyl group on the CL cell

increases. But above the immobilization time of 6 h, the CL intensity declined, probably because the denaturation of the cross-linking agent.

The optimized durations for enzyme and Au-Ag alloy NP immobilization are 3 h (Fig. 6) and 4 h (Fig. 7), respectively. There were no obvious distinctions in the CL peak intensity when the immobilization time was increased. It is indicating maximum absorption each of them on the CL cell.

< Fig. 5>

< Fig. 6>

< Fig. 7>

3.3. Optimization of chemiluminescence assay medium

3.3.1. Optimization of Cu^{2+} concentration

To investigate the optimum concentration of Cu^{2+}, the CL response of biosensor at constant concentration of [Emim][$EtSO_4$] in biosensor structure was measured against the varying concentration of copper ion. As it is obvious from Fig. 8, the CL intensity sharply increased with increasing concentration of Cu^{2+} until a concentration of 1.9×10^{-4} M is reached. The observed enhancement can be indicative of catalytic effect of [Emim][$EtSO_4$]/Cu^{2+} complex formed in the presence of phosphate [28]. However, further addition of Cu^{2+} revealed a gradual decrease of the CL intensity. This is most probably due to formation of insoluble copper (II) hydroxide at higher concentration, which can decrease efficiency of CL emission.

<Fig. 8.>

3.3.2. Optimization of Luminol concentration

As the luminescence reagent in this system, luminol concentration effects on the biosensor response. The effect of luminol concentration in the range of 2.5×10^{-4} to 2.5×10^{-3} M was investigated (Fig. 9). The result showed that increasing luminol concentration is followed by a steady increase in CL intensity. However, 2.5×10^{-3} M was chosen as the optimum concentration and higher concentrations were not evaluated, it could produce the saturation of the detector due to the high CL intensity of the blank signal obtained.

< Fig. 9>

3.3.3. Optimization of pH

In this biosensor, phosphate buffer solution is the medium of enzyme reaction and the CL reaction, and also is a essential medium for formation of [Emim][$EtSO_4$] /Cu^{2+} complex. Therefore, there are two factors that determine the overall response of the biosensor to pH: the influence of pH on the enzyme activity and the effect of pH on the generated CL signal. In view of the nature of luminol CL reaction, which is more desirable under basic conditions (pH 10–11), an alkaline medium would improve the sensitivity of the system. On the other hand, the optimal pH value for GOD is 6 to 7. The study of pH reveals the CL intensity was reached to the highest value at pH 7.5 (Fig. 10).

The [Emim][$EtSO_4$] /Cu^{2+} complex is an effective factor in the increasing kinetic of CL and enzymatic reaction, and consequently biosensor performance. The interaction taking place between Cu^{2+} and [Emim][$EtSO_4$] might be dependent on the presence of the labile proton in the C_2 position of the imidazolium ring, As Emim substituted at the C_2 position is advantageously used as reaction solvent for the Baylis—Hillman reaction [33] due to the fact that the conventional Emim can react with electrophilic reactants [34-36].

On this basis, it is presumed that the formation of [Emim][$EtSO_4$] /Cu^{2+} complex at approximately neutral pH can be main reason for decreasing the optimal pH.

< Fig. 10>

3.4. Binding of Glucose oxidase

As mentioned (see Section 2.7) affirmation of the immobilized glucose oxidase was possible by studying the fluorescence of released FITC in the solution. The spectrofluorimetric studies were carried out in released solution of CL cell surface after

treatment of the glass cells. As shown in Figure 11, at the FITC emission wavelength, significant fluorescence (λ_{em}=525) of the released solution was obtained .Therefore, binding of labeled enzyme to active glass cell and consequently the binding of the unlabeled enzyme can be proved. Control experiments showed that the procedure didn't interfere with the fluorescence of the label.

< Fig. 11>

3.5. Performance of biosensor for glucose measurements

The analytical performance of the proposed biosensor was examined under the optimum condition. The calibration graph of emission intensity (I) versus glucose concentration was linear in the range of 1.0×10^{-6} to 7.5×10^{-3} M, so 7.5×10^{-3} M is determined as the concentration of glucose that enzyme is saturated in Michaelis-Menten graph (insert in Fig. 12), and the detection limit was 4.0×10^{-7} M (S/N=3). The regression equation was $I = 1 \times 10^{+6} C + 14983$ (where C is the glucose concentration, M) with a correlation coefficient of 0.9975 (n = 10). A complete analysis, including sampling and washing, could be performed in less than 2 min with a relative standard deviation of less than 2% for 5.0×10^{-4} M glucose (n = 12). All those results indicated that the performance of the CL biosensor was compatible with those of the known and well assessed glucose CL sensors. The unique sensitivity, selectivity and stability of the proposed biosensor is related to suitable coupling of covalent link method with noticeable characteristics of chitosan and ion liquid at the synthesis NPs and binding of them to the cell surface that in addition to possibility improvement of CL and enzymatic kinetic, can provide efficient binding of the enzyme to CL cell and the simultaneously impact of nanoparticles and [Emim][$EtSO_4$] /Cu^{2+} complex on the enzymatic and chemiluminescence reaction.

< Fig. 12>

353 *3.6. Sample analysis*

354 To investigate the feasibility of the measurement system for analysis of glucose in
355 biological samples, glucose concentration in human serum and urine was examined. The
356 samples were collected from medical diagnostic laboratory and used as testing samples. The
357 fresh samples were first analyzed with a BT3000 Biochemical Analyzer, which used
358 spectrophotometric method combined with the enzymatic reaction of GOD and HRP [37].

359 The serum and urine samples were 10 and 100-fold diluted using distilled-deionized
360 water, respectively and analyzed without any pre-treatment with the proposed method. The
361 response of the sample solution was measured and compared with a set of glucose standard
362 solutions. The comparative results are shown in Table 1.

363 < Table 1.>

364 *3.7. Reusability and lifetime of biosensor*

365 Fig. 13 demonstrates the operational stability of the enzyme-CL biosensor for glucose
366 containing immobilized GOD in 0.1 M phosphate buffer at pH 7.5. Repeated measurements
367 with 1.0×10^{-4} M glucose solution were carried out. After each analysis, the immobilized
368 enzyme biosensor was washed with deionized water. The immobilized enzyme-biosensor
369 retained residual activity higher than 90% up to the 8th after 300 analysis and it was stable up
370 to analyse 570 samples during 4 months of operation at the end of which 50% of the initial
371 activity of the immobilized GOD-biosensor was retained. The relative activity of
372 immobilized GOD-biosensor at the first analysis was defined as control and attributed a
373 relative activity of 100%.

374 < Fig. 13>

375

376 The lifetime of biosensor depend mainly on the enzymatic stability and activity. The
377 long-term stability of the immobilized enzyme-biosensor was followed by two different

storage methods. One was immersed in 0.1 M phosphate buffer (pH 7.5) at 4 ◦C in refrigerator; another was stored at similar condition without buffer. Both of them were tested for storage stability. The obtained results were similar for them; the biosensors remained about 90% of its initial CL intensity, when they were stored in air at 4 ◦C for 2 months.

3.8. Interference studies

The effects of various interfering species which may accompany glucose in human serum and urine were also studied. Glucose (1×10^{-5} M) was placed in the reaction cell and increasing amounts of interfering substances were added into the solution. The maximum tolerable concentrations of coexisting substances are shown in Table 2, where the tolerance fold was defined as the maximum concentration of coexisting substances that caused a relative error <5% during the measurement of 10^{-5} M glucose solution. All species tested were tolerated at reasonable concentrations; confirm the high selectivity of the proposed method. Although, it is worth to mention that ascorbic acid at high concentration can influence at biosensor response, probably because ascorbic acid can easily reduce H_2O_2 produced from the enzymatic reaction. However, it doesn't have any interference at its physiologic normal level on the biosensor response to glucose.

< Table 2.>

4. Conclusions

A stable and sensitive glucose biosensor has been successfully fabricated by covalent immobilizing GOD via direct coupling of chitosan-induced Au/Ag alloy NPs dispersed in ion liquid using natural-polymer chitosan on the surface of CL cell. The immobilization of nano-alloy dispersed in ion liquid on biosensor and possibility of [Emim][$EtSO_4$] /Cu^{2+} complex formation between ion liquid on CL cell and copper ion in solution allowed high performance

of biosensor based on covalent-link in all key parameters such as reproducibility, reversibility, stability, response time and leaching characteristics -. Moreover, this biosensor offers low limit of detection, fast response time and long-term stability in comparison to some biosensors which used other supporting materials. A comparison of the main features of the present CL biosensor with others reported in the literature is given in Table 3. The innovative approach will be very effective at introduction of new methods for evaluation of other biological compounds by varying the enzymes.

< Table 3.>

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Figure1

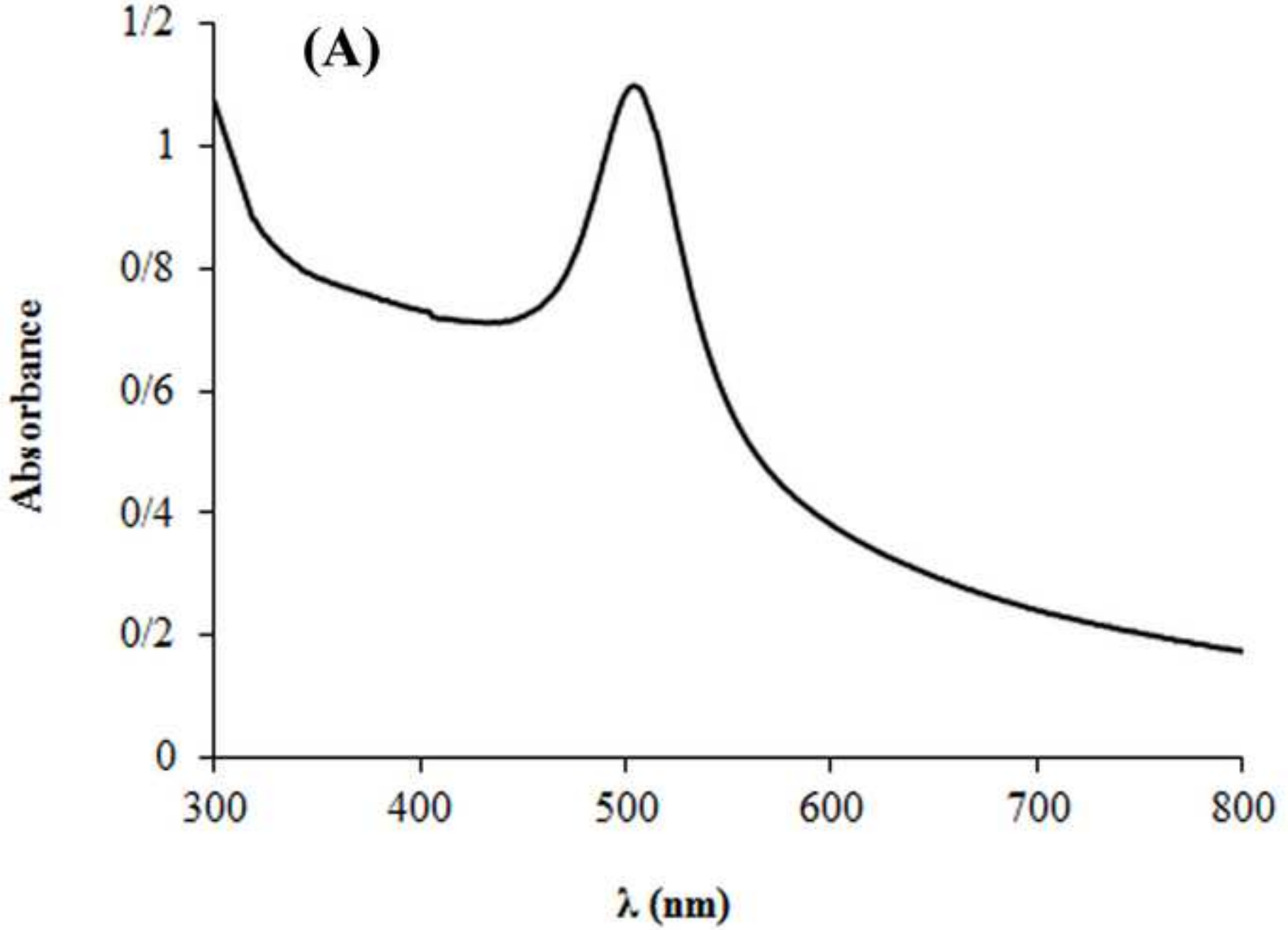
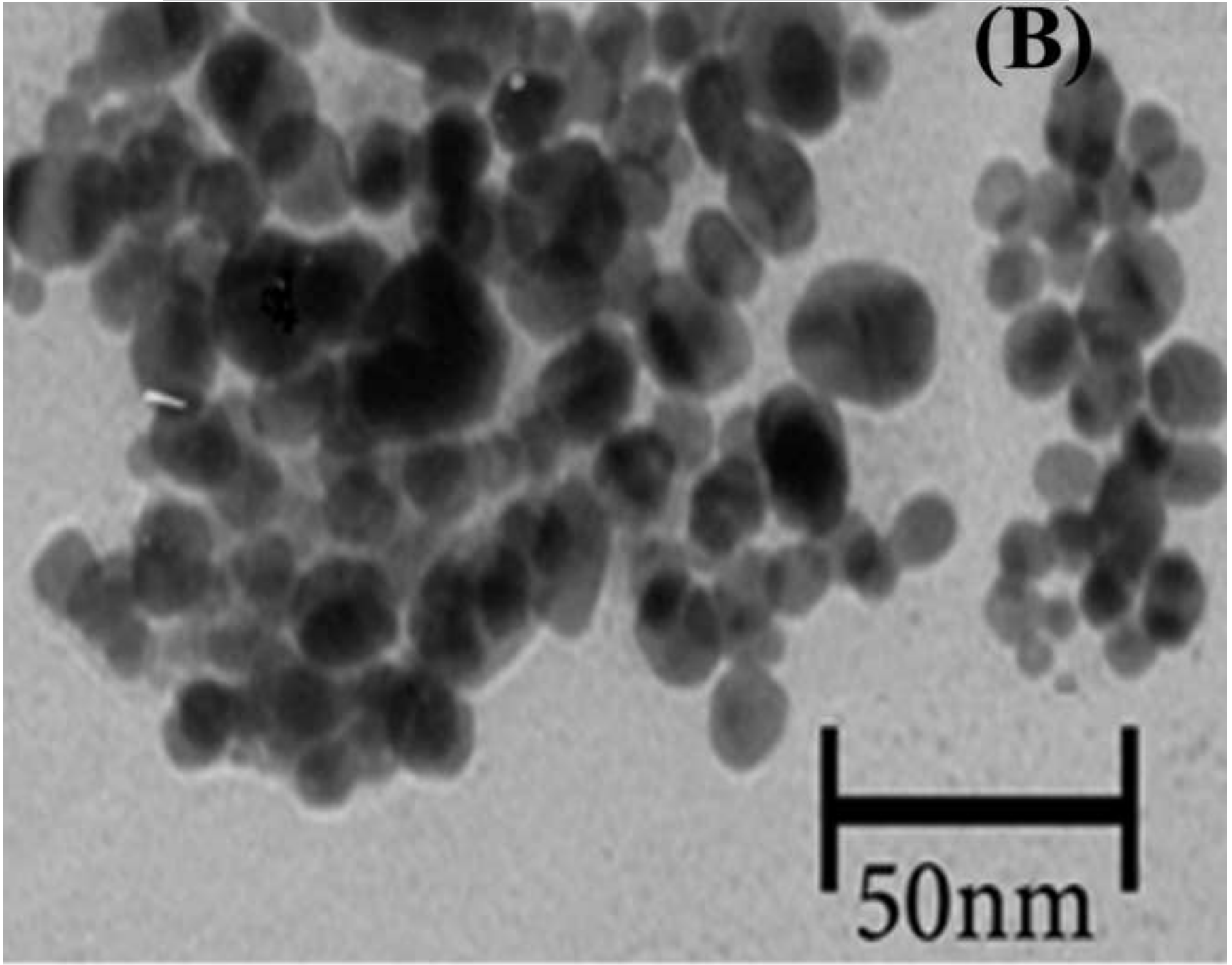


Figure1



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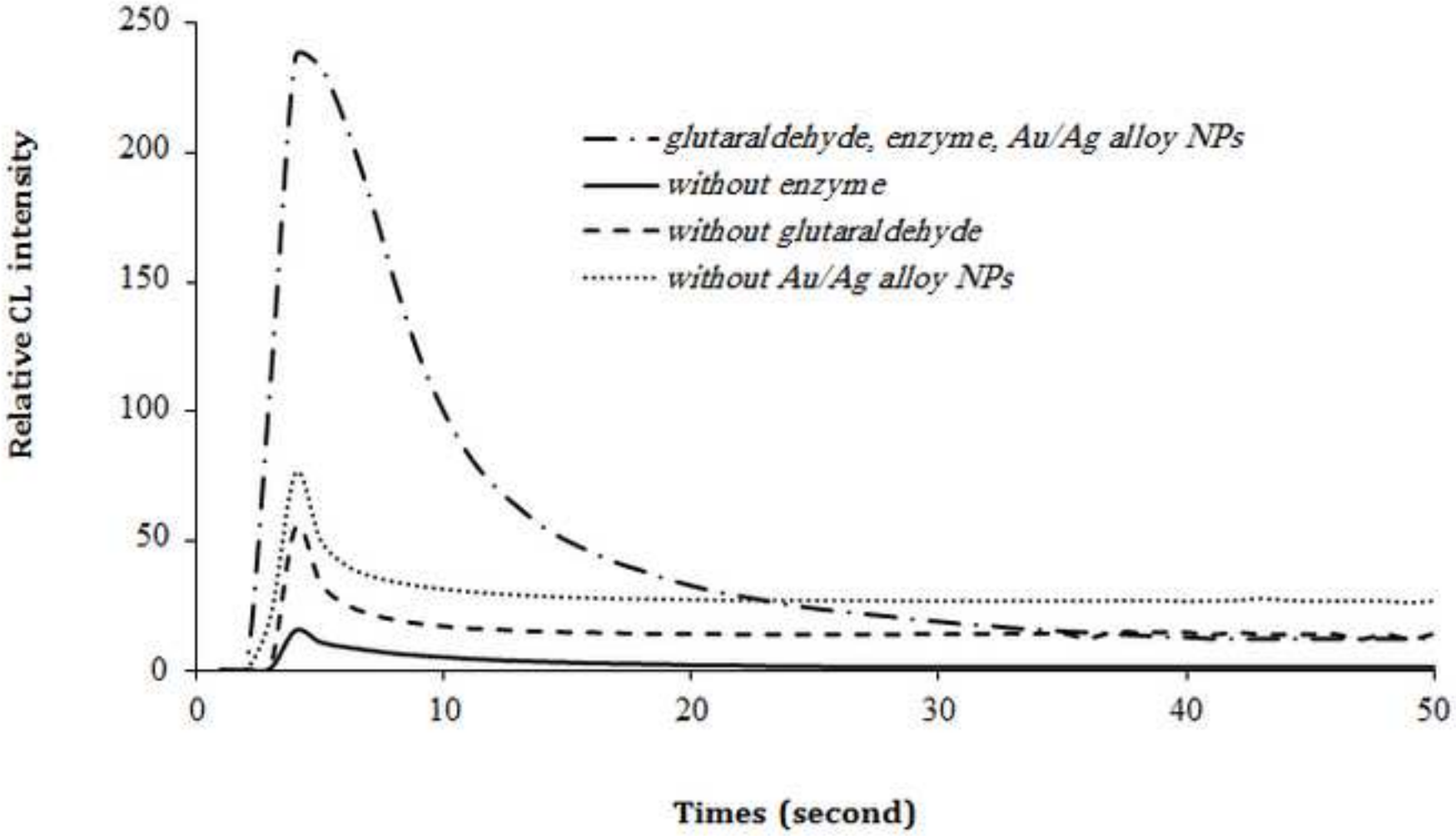

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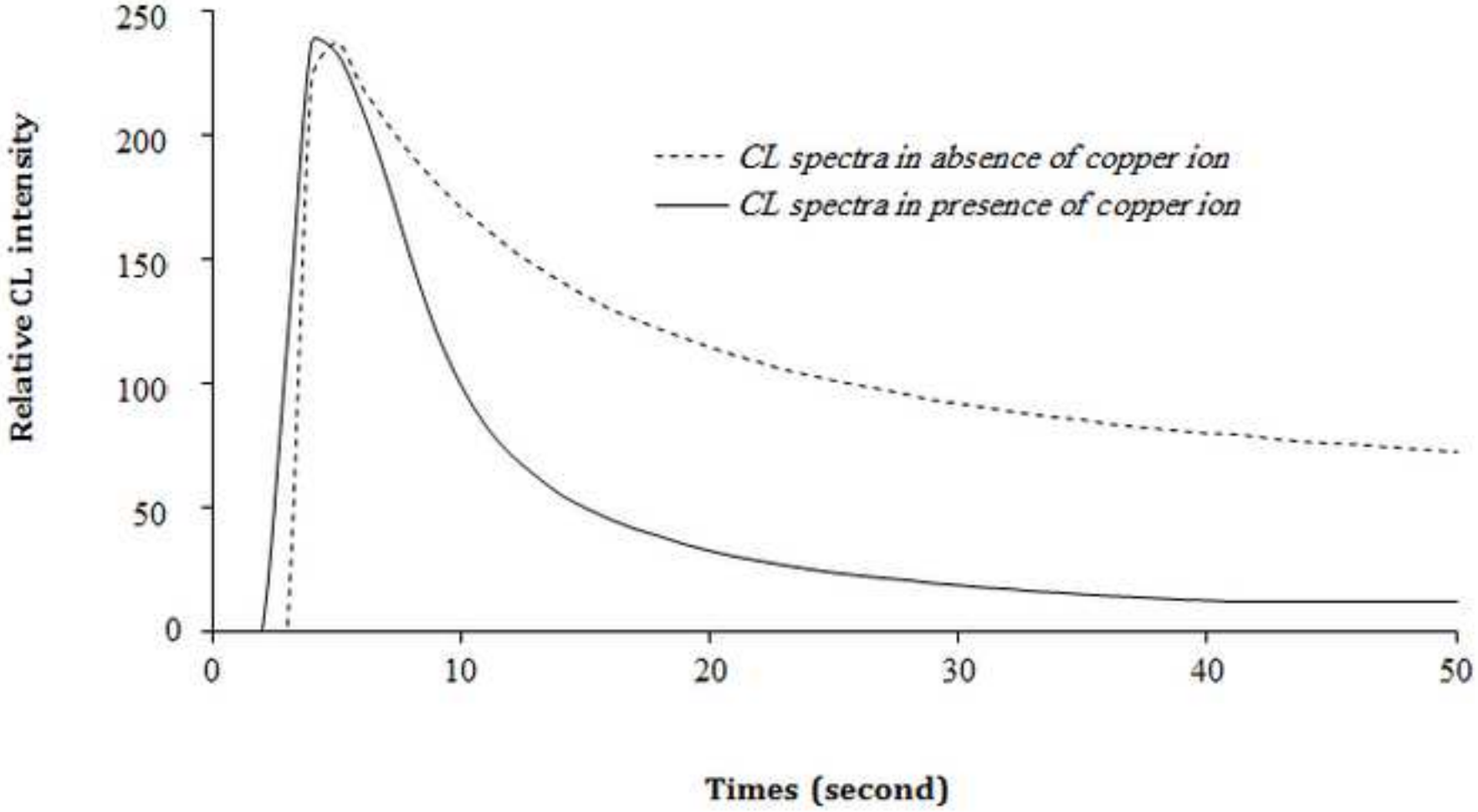

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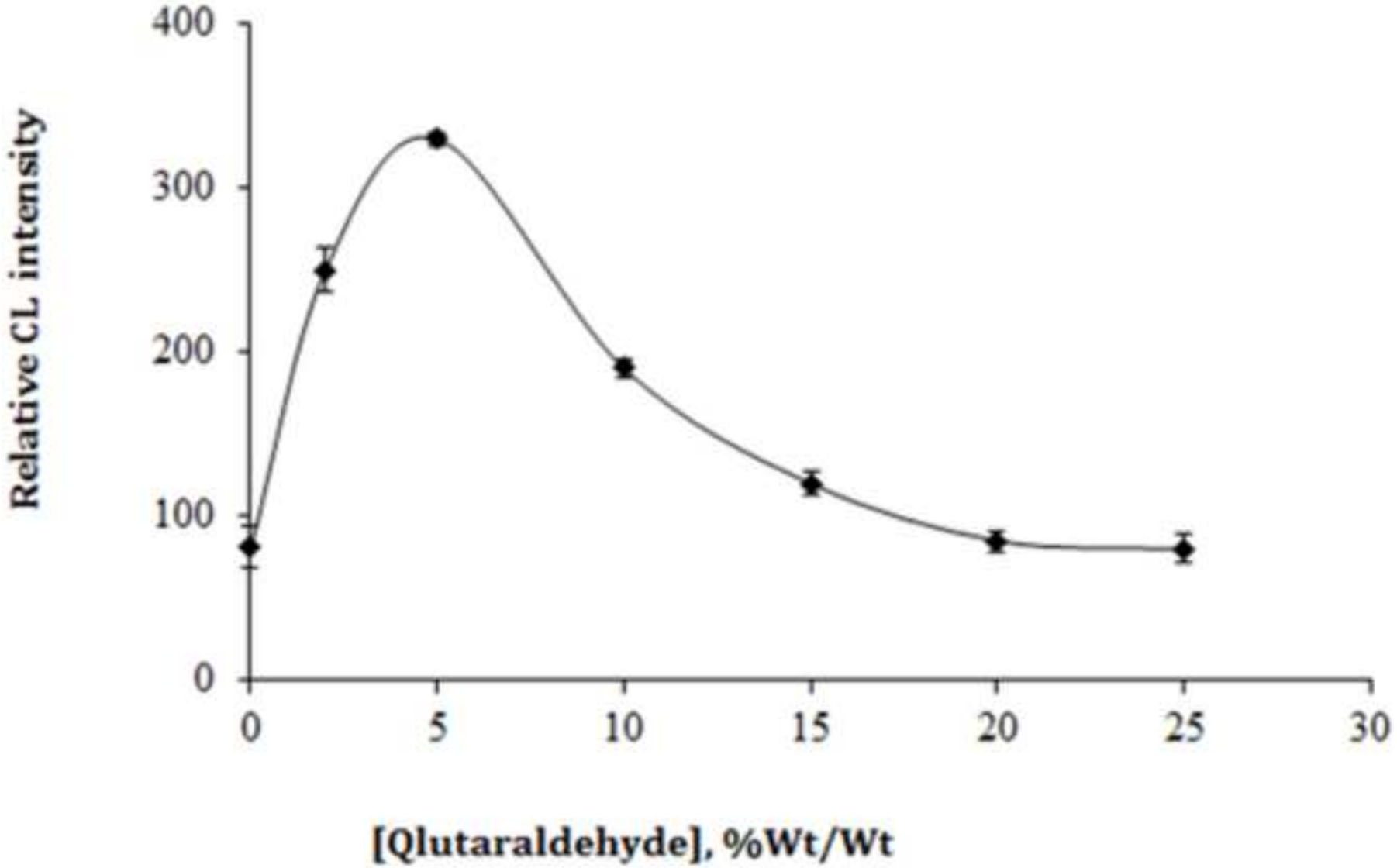

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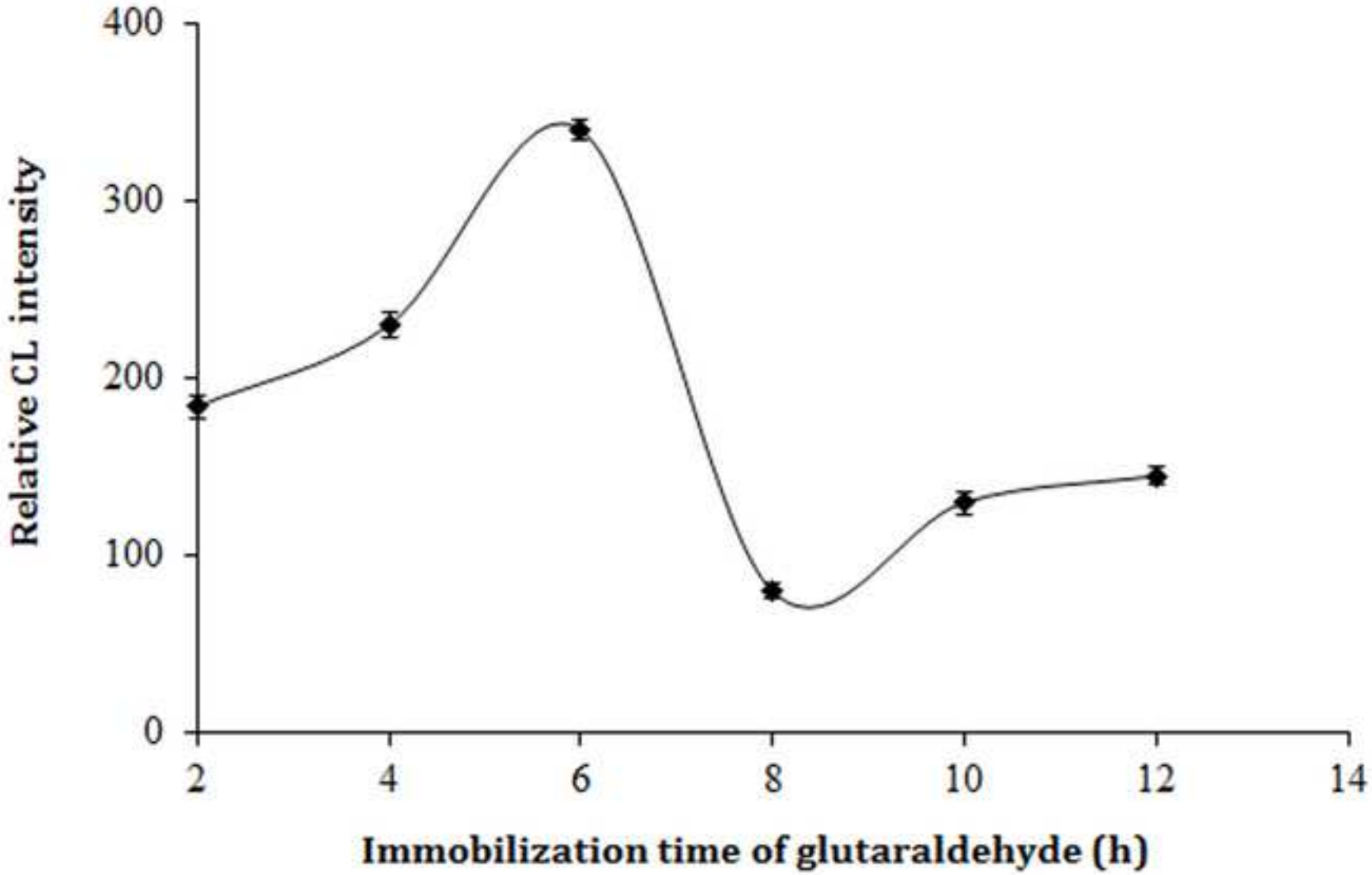

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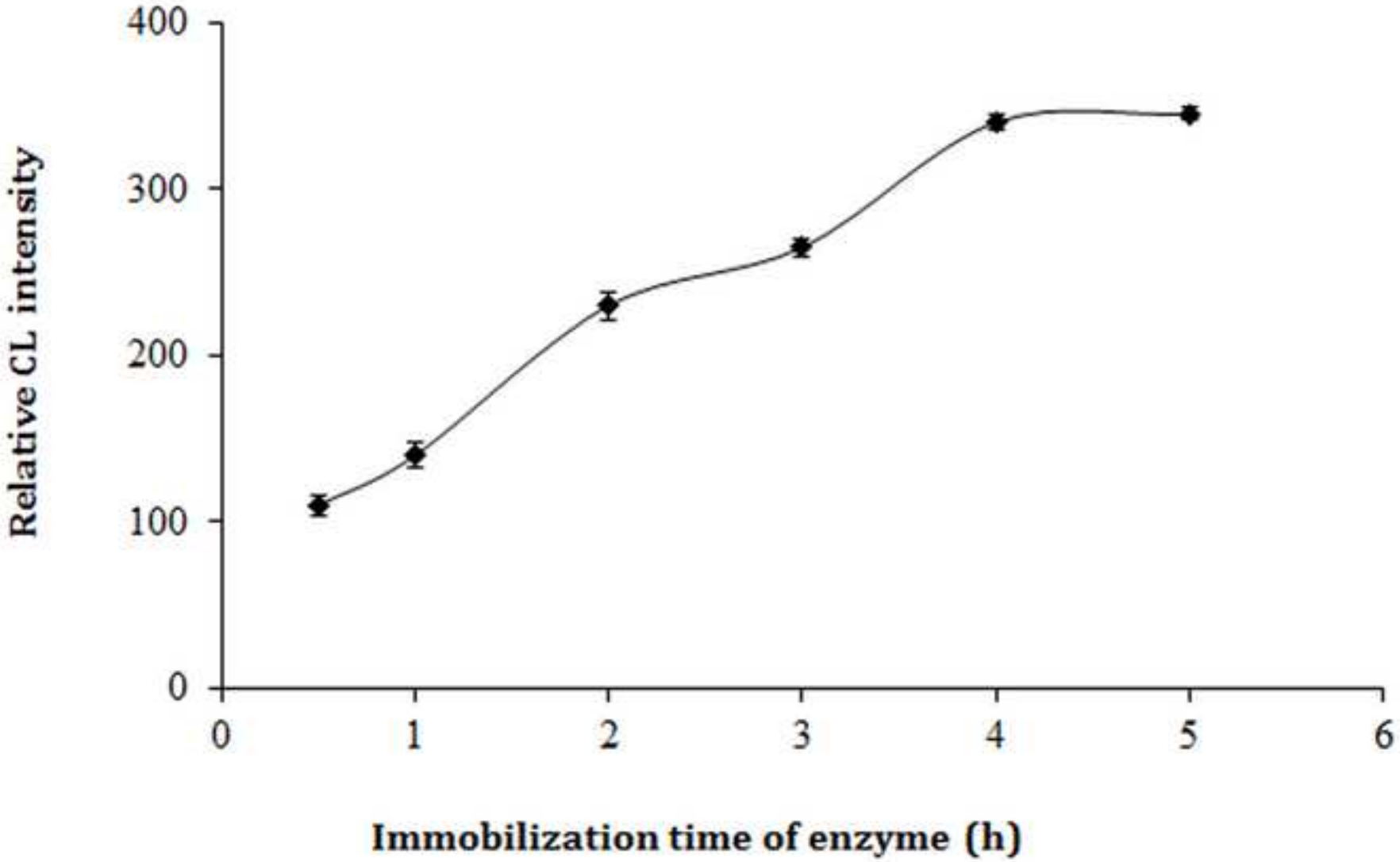

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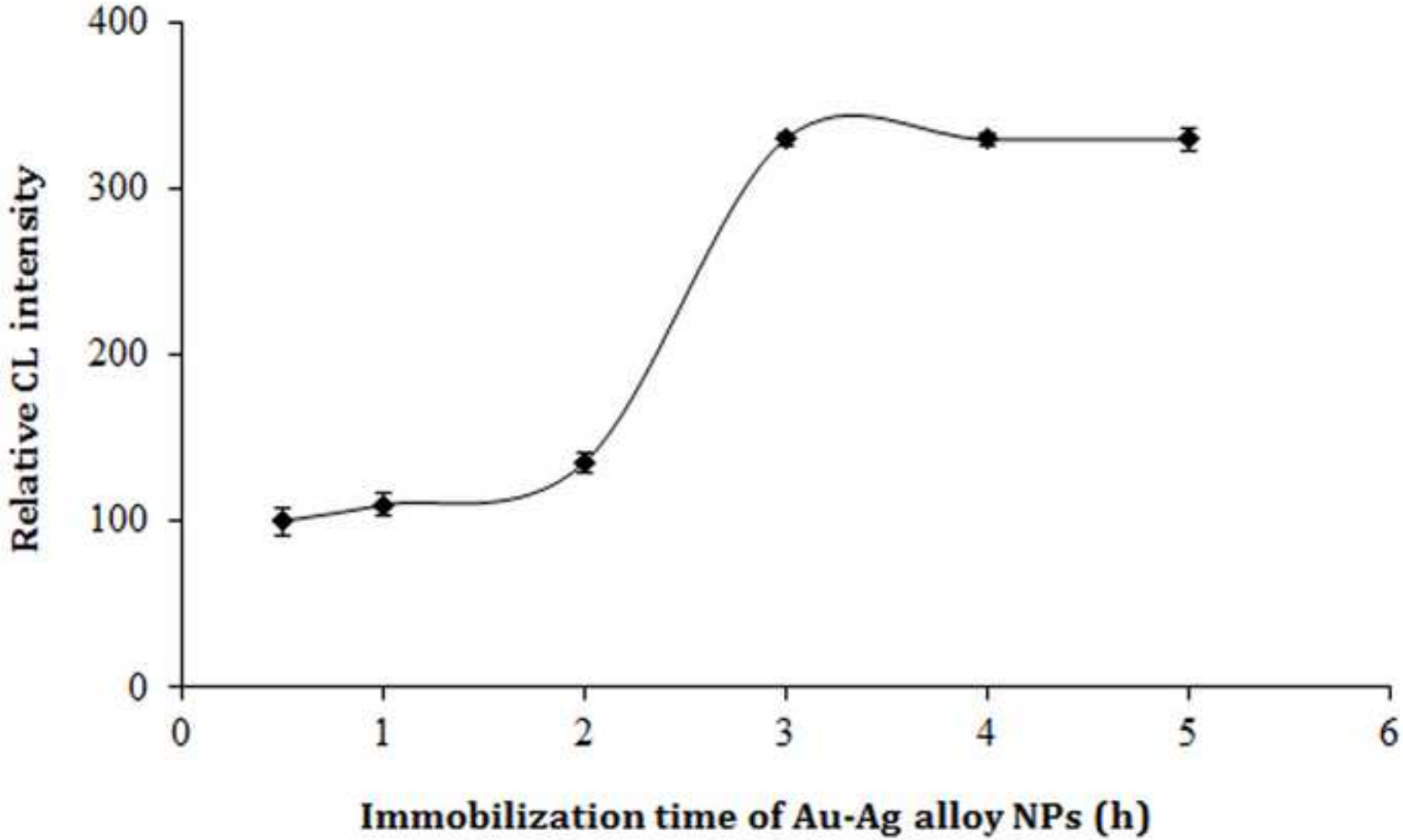

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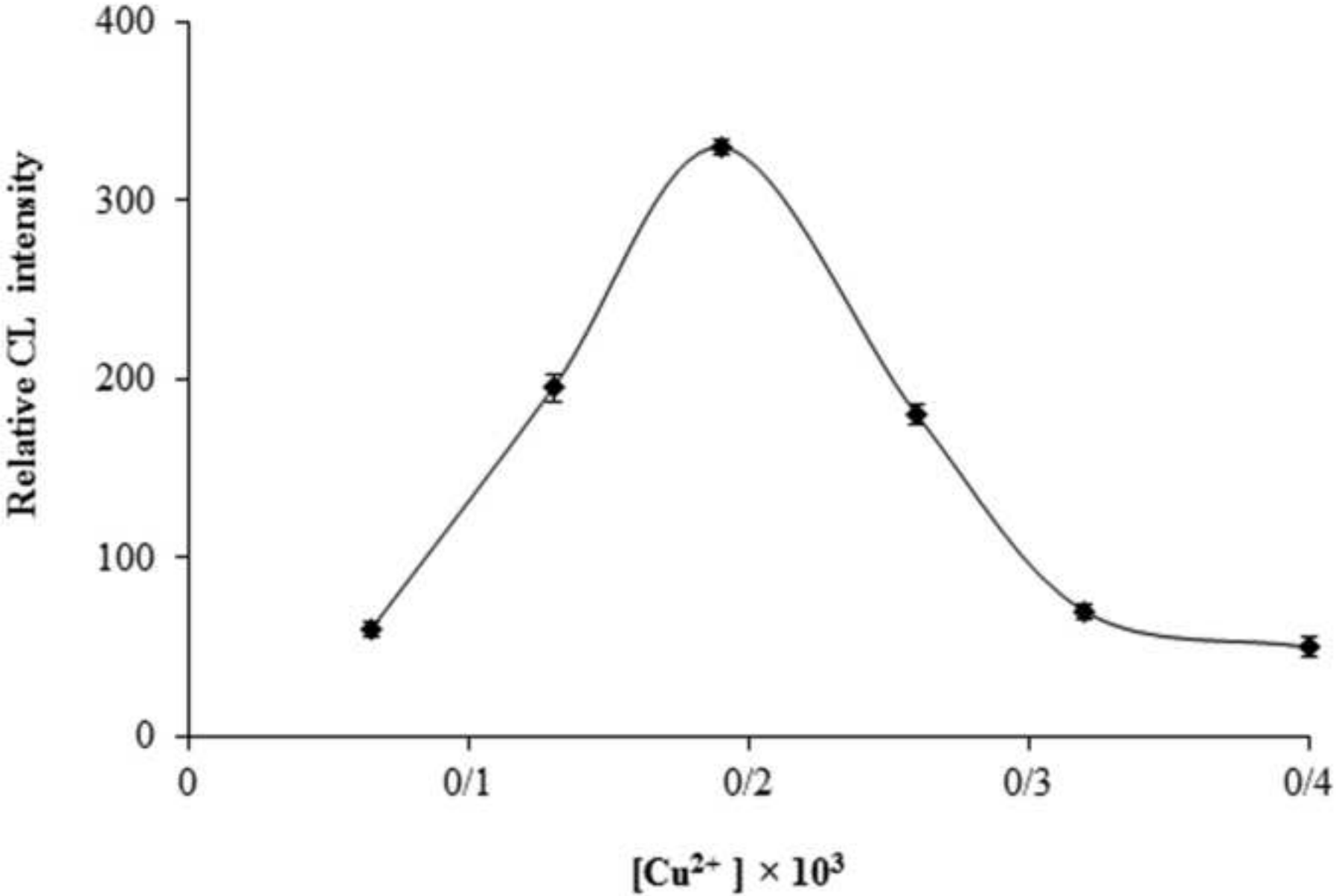

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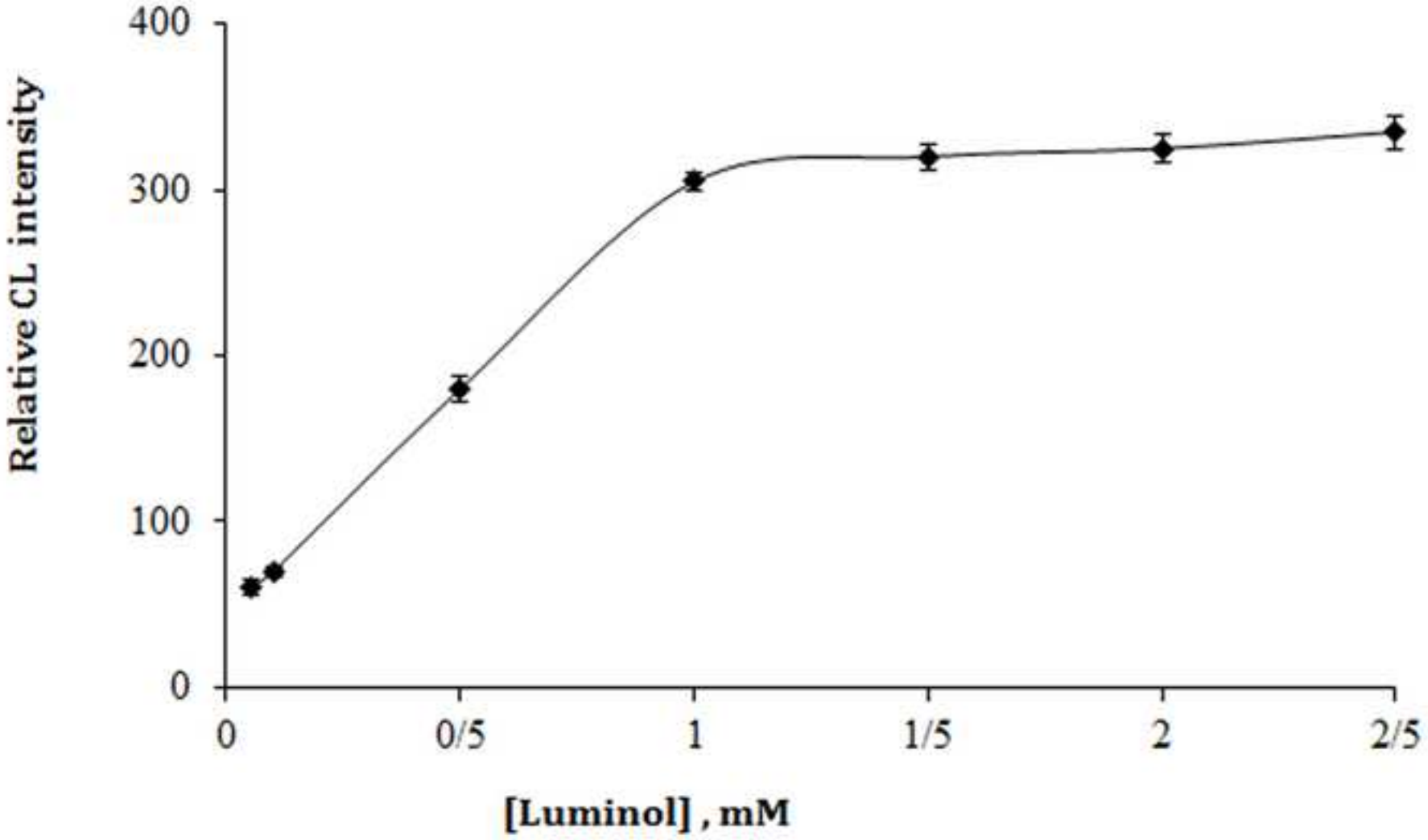

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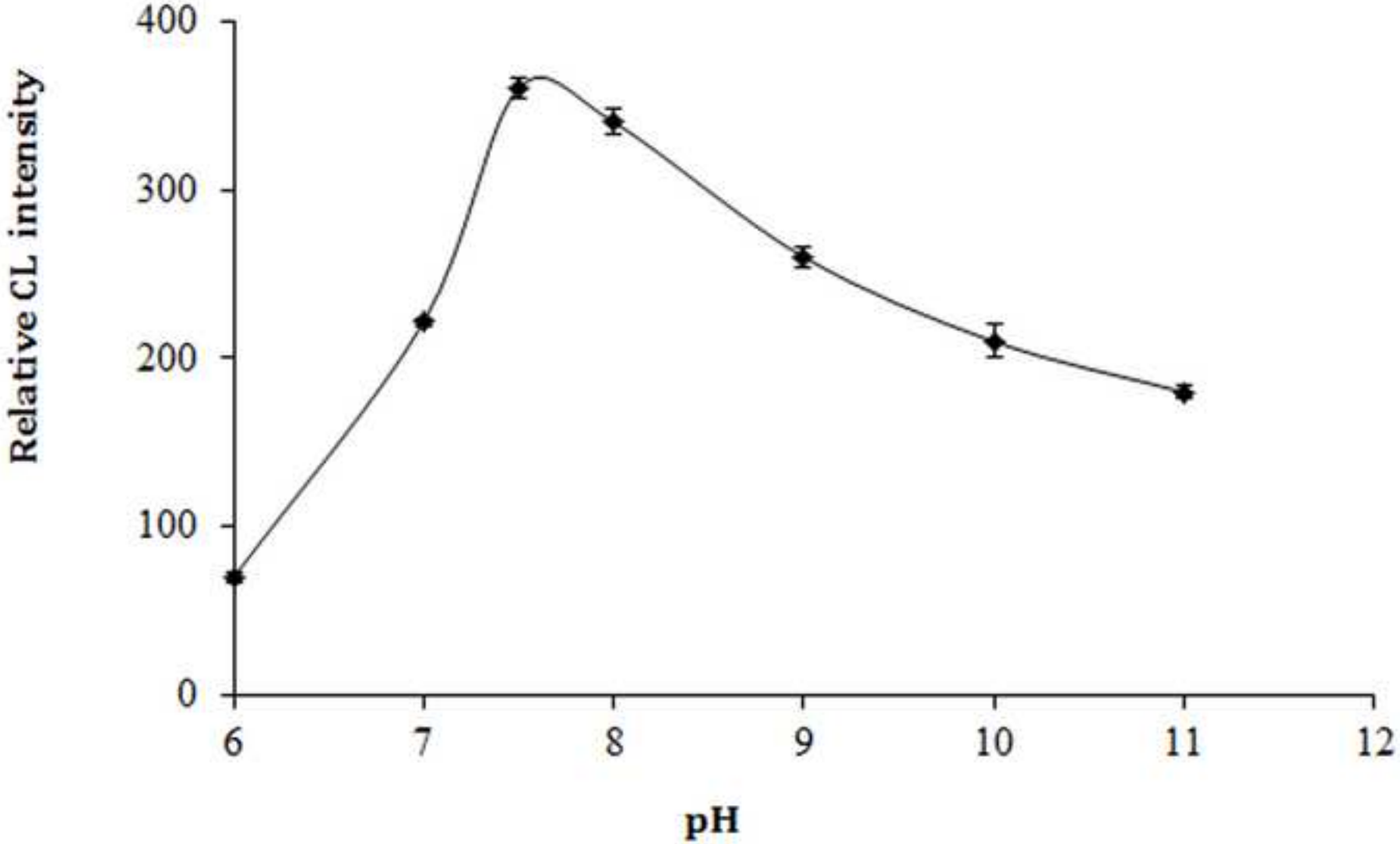

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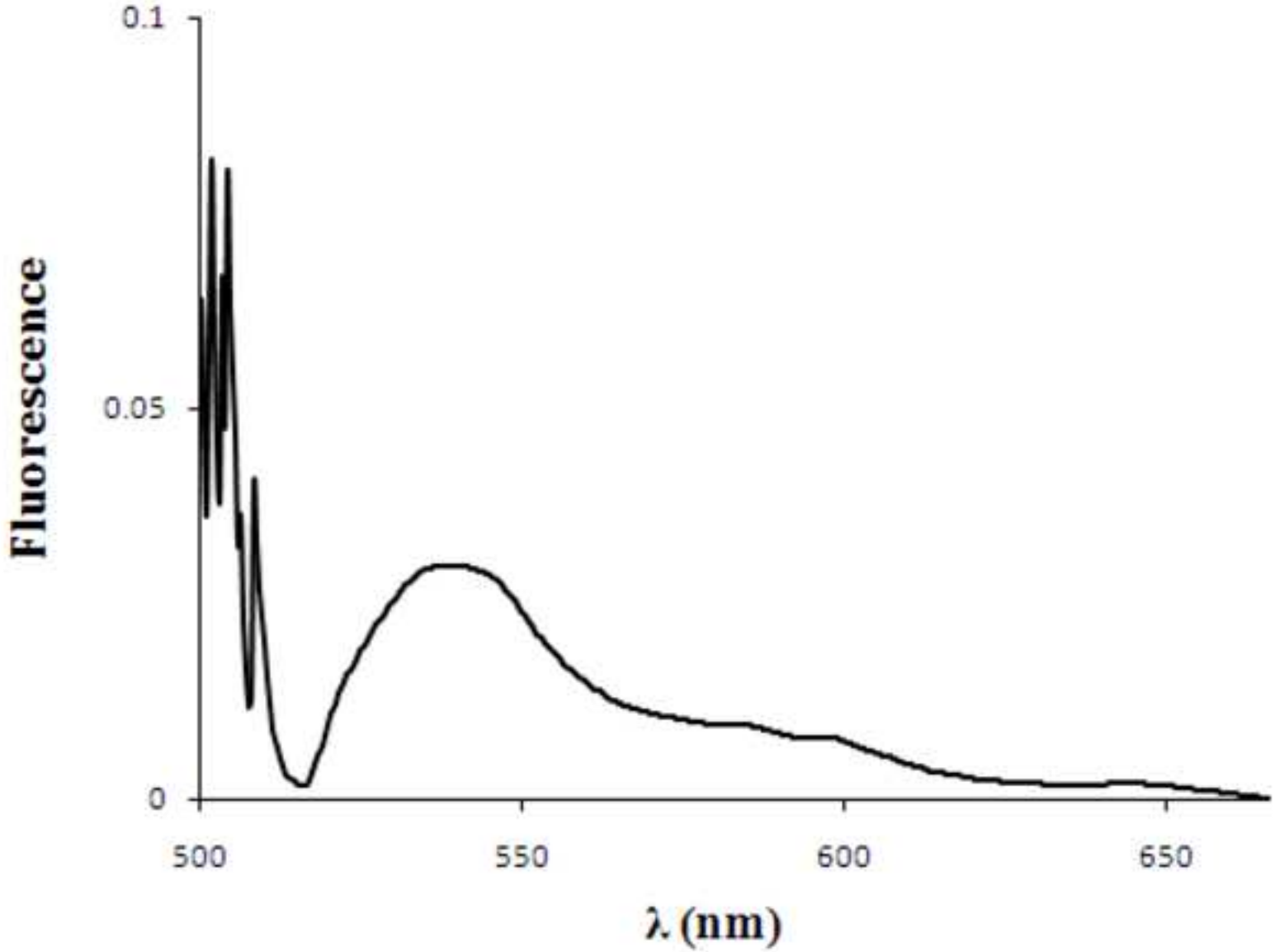

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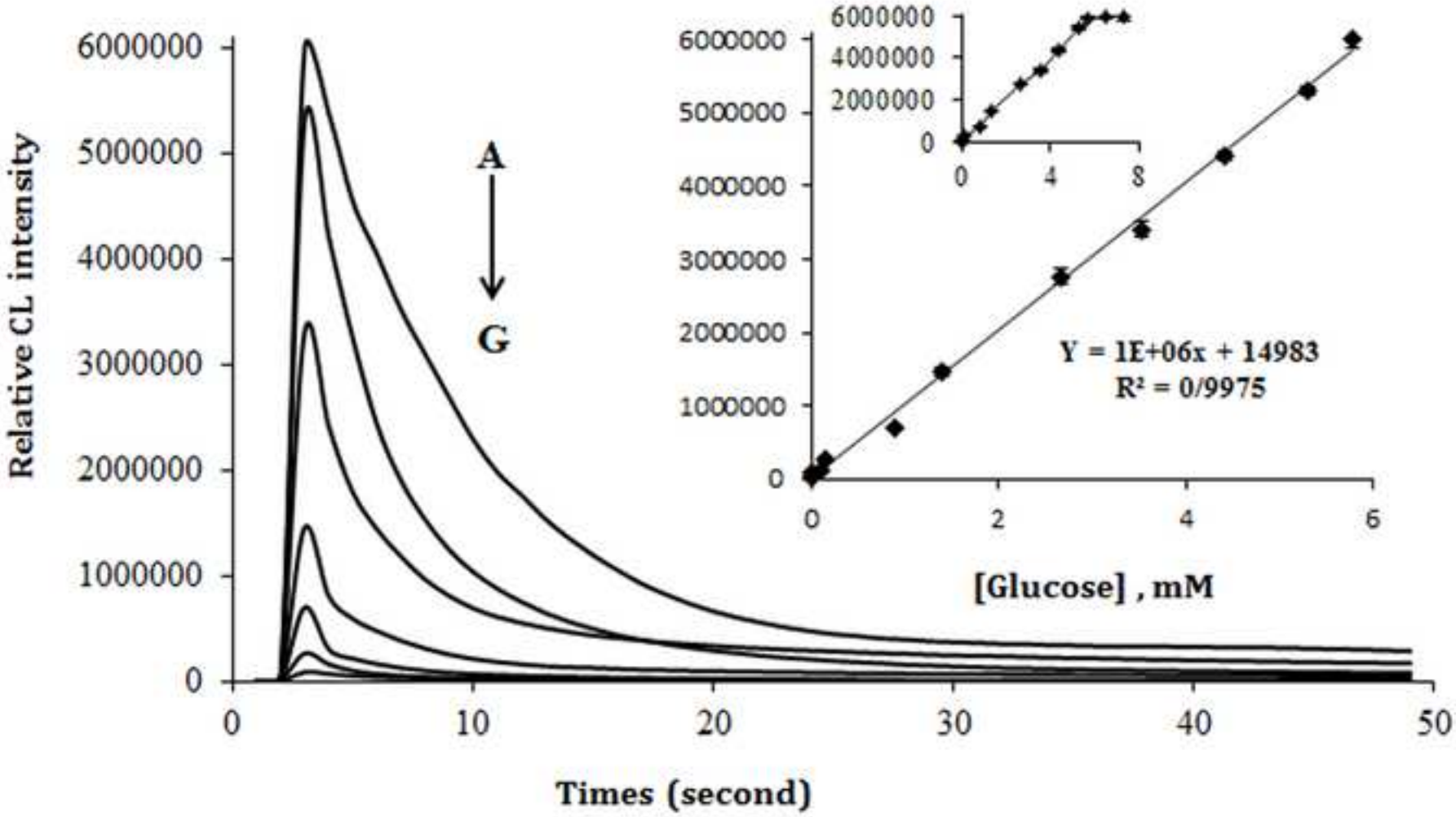

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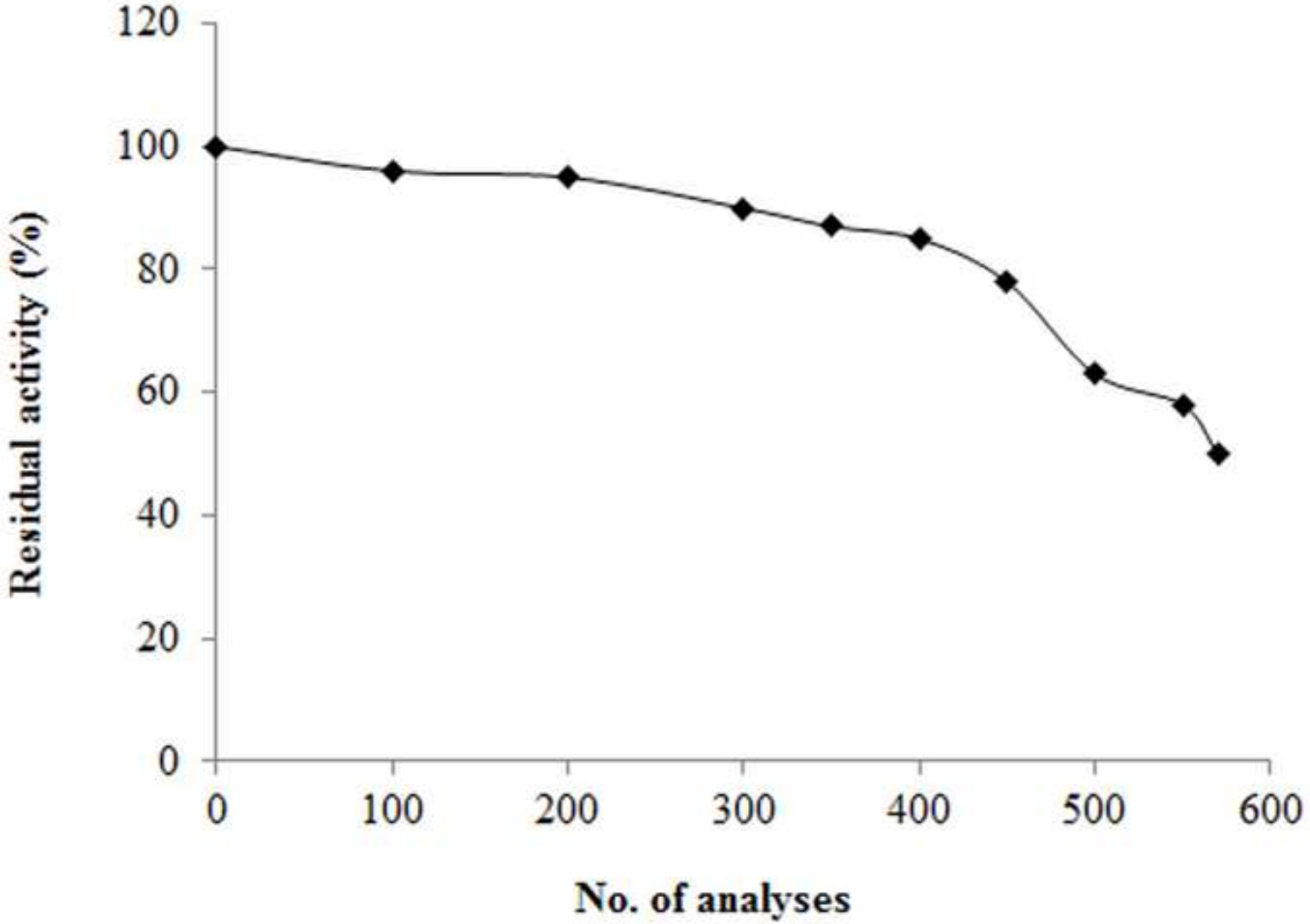

Table 1
Determination of glucose in serum and urine samples

Table 2
Tolerance folds Coexisting substances

Table 3
Comparison of the analytical parameters of the proposed biosensor with some reported methods for glucose determination

Table 1
Determination of glucose in serum and urine samples

Sample no.	Blood serum		Urine	
	CL method (mg/dl, n=3)	Photometric (mg/dl, n=3)	CL method (mg/dl, n=3)	Photometric[a] (mg/dl, n=3)
1	165.8±3.1	163	25.1±0.5	26
2	86.9±2.0	88	2.5±0.3	-
3	234.4±1.9	234	8.8±0.1	11

[a] LOD of the Pars Azmoon photometric kit is 5 mg dL−1.

Table 2
Tolerance folds Coexisting substances

Tolerance folds	Coexisting substances
≥1000	Na^{+}, K^{+}, Ca^{2+}, Cl^{-}, NO_3^{-}, PO_4^{3-}, SO_4^{2-}, starch
≥100	thiourea, urea and uric acid
≥50	bilirubin, sucrose, fructose and lactose
≥10	ascorbic acid

Table 3
Comparison of the analytical parameters of the proposed biosensor with some reported methods for glucose determination

Method	Linear range (M)	Detection limit (M)	Reference
Sol-gel /GOD- optical biosensor arrays	1.0×10^{-4}–5.0×10^{-3}	6.0×10^{-5}	[38]
C_{60} embedded in tetraoctylammonium bromide-ECL biosensor	5.0×10^{-7}–13.0×10^{-3}	1.6×10^{-7}	[39]
Gallium hexacyanoferratemodified carbon ionic liquid paste electrode	1.0×10^{-4}–6.0×10^{-3}	3.0×10^{-5}	[40]
Gold nanosphere –ECL biosensor	1.0×10^{-6}–4.3×10^{-3}	3.0×10^{-7}	[41]
Glucose oxidase/carbon-nanotube /NP in nafion film-CL biosensor	2.25×10^{-6}–1.75×10^{-4}	1.0×10^{-6}	[15]
Boronic anthraquinone derivatives/GOD- fluorescence sensor	8.0×10^{-5}–4.2×10^{-4}	1.1×10^{-5}	[42]
Photopolymerized fluorescence sensor	2.8×10^{-5}–5.5×10^{-3}	0.89×10^{-5}	[43]
Proteins modified with DNAzymes or aptamers/glucose biosensor	5.0×10^{-6}–2.0×10^{-2}	5.0×10^{-6}	[44]
This work	1.0×10^{-6}–7.5×10^{-3}	4.0×10^{-7}	-

Chitosan- induced Au/Ag alloy nanoparticles dispersed in ion liquid and its application in fabricating an ultrasensitive glucose biosensor based on Luminol-H_2O_2-Cu^{2+}/IL chemiluminescence system

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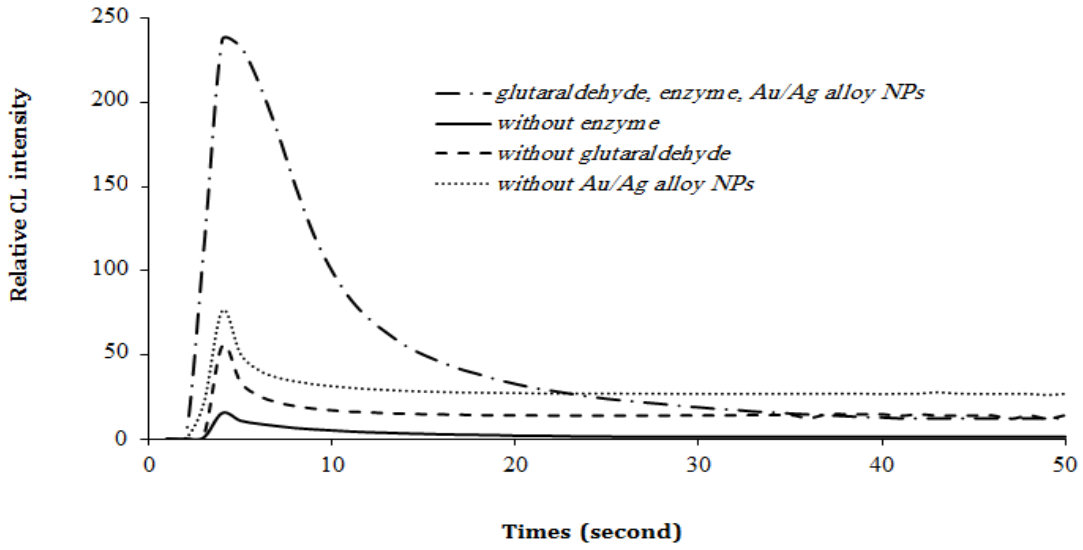

1- Chitosan-induced Au/Ag nanoalloy dispersed in IL was synthesised.

2- Glucose biosensor fabricated by covalent immobilization of enzyme and nanoalloy.

3- The nanoalloy catalyses enzymatic reaction and the CL reaction.

4- Immobilized IL on biosensor along Cu^{2+} in assay solution forms $[Cu^{2+}]$/IL complex.

5- $[Cu^{2+}]$/IL decreases the CL reaction pH and increases enzymatic reaction kinetic.