J Fluoresc (2015) 25:263–275
DOI 10.1007/s10895-015-1502-8

ORIGINAL PAPER

Evaluation of Luminol Chemiluminescence Based on Simultaneous Introducing of Coumarin Derivatives as Green Fluorophores and Chitosan-Induced Au/Ag Alloy Nanoparticle as Catalyst for the Sensitive Determination of Glucose

M. J. Chaichi · S. O. Alijanpour · S. Asghari · S. Shadlou

Received: 8 August 2014 / Accepted: 2 January 2015 / Published online: 3 February 2015

Abstract We report herein the development of a novel chemiluminescence system based on simultaneous introducing of synthetic coumarin derivatives and chitosan-induced Au/Ag alloy NPs on the luminol CL system and suggest how it may be useful for determination of glucose. Chitosan-induced Au/Ag nanoalloys in the coumarin derivatives intensified-luminol CL system, in addition to catalyze CL reaction can make a change in the process of coumarin derivatives effect as fluorophore on the luminol CL system. This phenomenon is caused by interaction between active functional groups of coumarin derivatives and chitosan. The interaction strength depends on the coumarin derivatives' structure and their substituents. Considering the inevitable trend luminol radical and superoxide anion radical to absorption on the surface of the embedded Au/Ag nanoalloy in the chitosan matrix, it can be concluded that chitosan acts as a platform for all reagents involved in the CL reaction including coumarin derivatives, Au/Ag nanoalloy and luminol, and electron-transfer taking place on it; Placing all chemiluminescent reagents together on the chitosan network can lead to a powerful CL due to increasing rigidity of CL system. The most efficient coumarin derivative on the Au/Ag nanoalloy-fluorophore-luminol-H_2O_2 CL system, in relation to interaction capability with chitosan' functional groups, was selected and the CL condition in presence of it was optimized. Whereas the glucose oxidase-mediated oxidation of glucose yields gluconic acid and H_2O_2, under optimum condition the most efficient CL system was applied to detection of glucose due to enzymatically production of hydrogen peroxide. The linear response range of 1.5×10^{-6}–5.0×10^{-3} M and the detection limit (defined as the concentration that could be detected at the signal-to-noise ratio of 3) of 7.5×10^{-7} M was found for the glucose standards. Also, the developed method was successfully applied to determination of glucose in real serum and urine samples of diabetic patients and validated against colorimetric spectroscopy method.

Keywords Chemiluminescence · Luminol · Fluorophore · Au/Ag alloy NPs · Glucose

Introduction

The glucose constitutes the main source of energy in living organism [1]. Levels of glucose in tissues and biological fluids are strictly controlled by the homeostatic balance within the definite range and can be changed at some pathology, e.g. at diabetes. Therefore, a large number of efforts have been focused on developing effective diagnostic tools for the benefit of diabetic patients in recent years. [2–9]. To date, the most common glucose determination methods are based on the electrochemical or spectrophotometrical detection of hydrogen peroxide liberated in enzymatic reaction between glucose oxidase (GOD) and glucose [10–18]. However, both methods are subject to several interferences. These interferences include reducing substances, such as bilirubin, ascorbic acid, uric acid, and drug metabolites [11, 19, 20]. In addition, these methods are deemed tedious and suffer of drawback such as low sensitivity or limited linear range. Other disadvantages of these methods are costly, time-consuming and analytically troublesome [21, 22]. These problems seriously limit their application for glucose determination in physiological

M. J. Chaichi (✉) · S. O. Alijanpour · S. Asghari · S. Shadlou
Department of Chemistry, University of Mazandaran, Babolsar, Iran
e-mail: jchaichi@yahoo.com

samples. Hence, developing a novel sensitive, rapid, simple and cheap method for glucose determination in biological fluids in the presence of other species would show appealing alternative. Recently, chemiluminescence (CL) has attracted extensive interest to glucose measurements, owing to its wide linear range, inexpensive apparatus, fast response time, no disturbance from background scattering light, low detection limit, high sensitivity and wonderful reproducibility [23–25]. But here the encountered problem is that this kind of enzymatic reaction must be carried out at neutral medium (about pH = 7), whereas the CL is efficient only in alkaline medium [17, 25]. This contradiction can reduce the efficiency and sensitivity of method. One possibility how to achieve pH optimum for enzymes generating hydrogen peroxide despite the high sensitivity of CL method is to perform the analysis in a neutral medium simultaneously using the amplification of chemiluminescence reaction.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which is a versatile and widely used chemiluminescence reagent, has been observed to produce CL emission at a wavelength of 425 nm under many conditions. Luminol CL has some limitations such as weak intensity, quick declining, discontinuous emission and short emission band (which are not sensitive to most photon detectors) [26–28]. Apart from this, CL spectra can be obtained in two methods. Sometimes they record by the "fast scanning" mode of a fluorescence spectrometer (such as PE LS-55, 1500 nm/min) [29]. It is based on the scanning process as quickly as possible, thus it may lose sensitivity and cannot completely overcome the difficulty to capture the spectrum of quick and discontinuous CL emission. Another method is based on scanning of CL intensities while fluids flow and mix in a tube [30, 31] to obtain stronger and more stable CL signals, but the spectral resolution is quite low and high random error cannot be avoided.

CL resonance energy transfer (CRET) is a non-radiative energy transfer from a CL donor to a suitable acceptor [32]. A CL donor in exciting state can transfer energy through the chemically initiated electron exchange luminescence (CIEEL) mechanism [33] to an acceptor chromophore in close proximity, and indirectly liberating "light". CRET, which occurs by oxidation of a luminescent substrate without an excitation source, could reduce dramatically the background autofluorescence and fluorescence bleaching, and enhance CL emission of luminol [32, 34]. Xu and coworkers demonstrate that the suitable fluorescence dyes as fluorophore are powerful tools to enlarge CL signals of luminol and do not join the CL reactions. In this situation, the overall quantum yield of luminol-CL reactions is directly dependent on the fluorescence quantum yield of the Fluorophore [32].

In recent years, nanomaterials with unique redox catalytic properties have gained increasing attention as a novel alternative to catalyze redox CL reactions, providing amplified CL emission. Many investigations have indicated that the use of metal or semiconductor nanoparticles in CL reactions could provide new approaches to enhance the inherent sensitivity and expand new applications of this mode of detection [35]. Luminol–metal nanoparticle is one of classic CL systems, and some CL analytical methods are known to be based on the catalysis of the oxidization of luminol with metal nanoparticle. In these systems, nanoparticles can participate in CL reactions as reductants, catalysts, luminophors and even a surface that electron-transfer processes taking place on it. For example, Cui et al. have reported many prominent works about noble metal nanoparticles-catalyzed luminol CL systems. It been demonstrated that gold, silver and platinum nanoparticles could greatly enhance a series of CL reactions including luminol-H_2O_2 [36], luminol-$K_3Fe(CN)_6$ [37], luminol-$AgNO_3$ [38], luminol-hydrazine [39]. These nanoparticles can adsorb H_2O_2 and luminol on the surface of them to facilitate the CL reaction [40, 41]. Ag and platinum Nanoparticles exhibit the stronger CL catalytic activity than Au NPs, although platinum NPs is very expensive [42]. Moreover, Au nanoparticle is an efficient catalyst at enzymatic activity without degradation effects on enzyme [43, 44]. Therefore, synthesis of Au/Ag nanoalloy can be useful to catalyze the enzymatic CL reaction. It is worth to mention Au/Ag alloy formation takes place easily than the other systems cause of the lattice constants of these metals are nearly the same and the miscibility gap between Au and Ag also less [45].

It suggests the simultaneous introducing of fluorophores and nanometals in the luminol CL system with innovative method might have potentials to be applied for the purpose of higher sensitivity in trace analysis.

Chitosan is a type of natural polyaminosaccharide with network structure, synthesized from the deacetylation of chitin, [46] that can help us for this purpose. Chitosan can be used as an adsorbent to remove heavy metals and dyes due to the presence of amino and hydroxyl groups, which can serve as the active sites [47]. It has some other interesting properties: chitosan can produces some metal NPs by acting as stabilizer and reductant [48], it has been widely used as the immobilization matrix for a large number of compounds, and even it could provide a suitable microenvironment similar to the native environment of the biomolecules to retain their [49–51]. Recently, gold NPs-chitosan has been widely used not only as the immobilization matrix of HRP but also as the enhancer of luminol CL reaction [52]. According to the above mentioned; the chitosan's properties and nature of the good biocompatibility and miscibility of Au and Ag NPs [42], it seems synthesis and introducing of chitosan-induced Au/Ag alloy NPs on the fluorophore-luminol-H_2O_2 CL system can be valuable in improving efficiency of the CL system.

In the present study, five coumarin derivatives of dimethyl 2-(tert-butylcarbamoyl)-3-(4-(trifluoromethyl)-2-oxo-2H-chromen-7-ylamino)succinate (A), di-tert-butyl 2-(tert-butylcarbamoyl)-3-(4-(trifluoromethyl)-3,4-dihydro-2-oxo-

2H-chromen-7-ylamino)succinate (B), di-ethyl-2-(tert-butylcarbamoyl)-3-(4-(trifluoromethyl)-3,4-dihydro-2-oxo-2H-chromen-7-ylamino)succinate (C), dimethyl 2-(cyclohexylcarbamoyl)-3-(4-(trifluoromethyl)-2-oxo-2H-chromen-7-ylamino)succinate (D) and di-tert-butyl 2-(cyclohexylcarbamoyl)-3-((4-(trifluoromethyl)-1,2-dihydro-2-oxonaphthalen-7-yl)methyl)succinate (E) were synthesized (Fig. 1) and introduced as new efficient fluorescence brighteners (acceptor) of the CRET process with the chemiluminescence of luminol as donor. At following, the ability of chitosan-induced Au/Ag alloy nanoparticles in improving efficiency of fluorophore-luminol– H_2O_2 CL system and how it does has been described. Finally, the CL system in presence of the most efficient fluorophore was used to detect H_2O_2 generated in enzymatically oxidation of glucose and consequently glucose measurement in the standard and real samples.

Materials and Methods

Reagents

Glucose, $HAuCl_4 \cdot 4H_2O$ (48 % *w/w*), $AgNO_3$, hydrogen peroxide (30 %), sodium phosphate monobasic monohydrate (99 %) and disodium hydrogenophosphate (99 %) were supplied from Merck, Germany. Chitosan flakes (high molecular weight, 85–90 % deacetylation) were purchased from Aldrich. Glucose oxidase (GOD) solution (1000 U/ml) was obtained from Molecular Probes (Zist chemistry, Tehran, Iran). A 0.02 M stock solution of luminol (Fluka) was prepared in 0.10 M NaOH and diluted to deionized water before use. 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.

The fluorophores were synthesized and then purified in organic laboratories [53]. The preparation details of polyfunctionalized coumarin rings are as follows; the three-component reactions involving 7-amino-4-(trifluomethyl) coumarin as an *NH*-acid and acetylenic esters in the presence of alkyl isocyanides. The preparation mechanism of coumarin derivatives is shown in Scheme 1. Fluorophores were dissolved in methanol for use.

Instrumentation

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 instrument, respectively.

Procedures

Chemiluminescence assays were performed in a 0.1 M phosphate buffer (pH = 8) with a 550 μL final volume. Briefly, cells

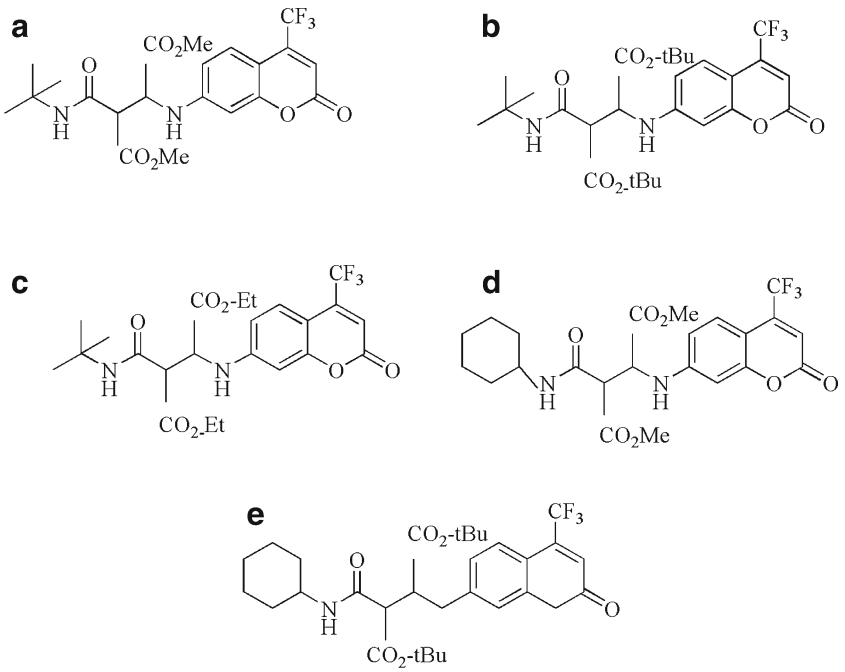
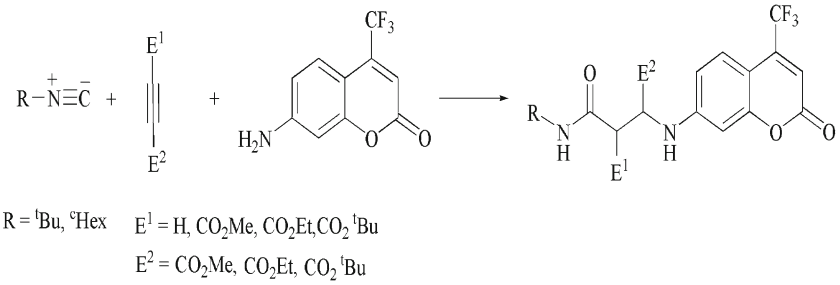


Fig. 1 Molecular structures of coumarin derivatives

Scheme 1 The preparation mechanism of coumarin derivatives



containing buffer were filled with 300 μL of solutions of luminol, fluorophore, and chitosan-induced Au/Ag alloy NPs (if needed) at particular concentrations and 150 μL of a solution of complementary reagents (various concentrations of hydrogen peroxide) were injected to initiate the light emission. The signal increases rapidly for 2 s to attain its maximum value and then decreases gradually and becomes constant after 10 s.

Complementary reagents for the glucose assay were produced by the following method: 250 μL of glucose of an appropriate concentration (in 0. 1 M phosphate buffer pH 7.5) was transferred into a homemade glass reactor designed for catalytic oxidation of glucose. Then, 250 μL of glucose oxidase of an appropriate concentration (in 0.1 M phosphate buffer pH 7.5) was transferred into the glass reactor. The cell was closed and the contents were stirred for 2 min. then the stirring was stopped and 150 μL of the resulting solution was injected to the CL measurement cuvette.

Synthesis of Chitosan-Induced Au/Ag Alloy NPs

The chitosan- induced Au/Ag alloy NPs based on microwave method [54] and our previous experience were synthesized [55]. 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 procedure for preparation of the Au/Ag NPs, 2.5 ml of both $HAuCl_4$ and $AgNO_3$ (5 mM) were mixed and sonicated for 30 min in order to the metal ions distribute in the solution homogeneously. Then, the mixture were added to the 1 % acetic solution of CS (50 ml, 1 mg/ml) in a 250-ml glass flask (pH = 4–5), which was placed in a microwave oven and connected to a condenser. The reaction mixture was irradiated by microwave in a predesigned mode (200 W–30 min). After microwave irradiation treatment, the yielding brown-red colloidal solution was naturally cooled to room temperature. Finally, the cooled solution was stored at 4 °C in the refrigerator until use. The nanoalloy formation is confirmed by the UV–Visible absorption spectra (Fig. 2a) and TEM data (Fig. 2b). Statistical analysis of TEM data revealed that the average diameters of Au/Ag alloy (3:2) nanoparticles [56] were 12.0± 2.0 nm.

Result and Discussion

Study of Coumarin Derivatives and Chitosan-Induced Au/Ag Alloy NPs on the H_2O_2-Luminol CL System

Figure 3 shows emission spectra of the coumarin derivatives. Considering intensive emissions at wavelength range 475–479 nm for the coumarin derivatives, the general effects of their on H_2O_2-luminol CL system were evaluated. As expected, an enhancement of the CL intensity was occurred in the presence each of them. While, a weak CL signal was observed in the absence of them (Fig. 4a). It is expected coumarin derivatives behave as a fluorophore on the H_2O_2-luminol CL

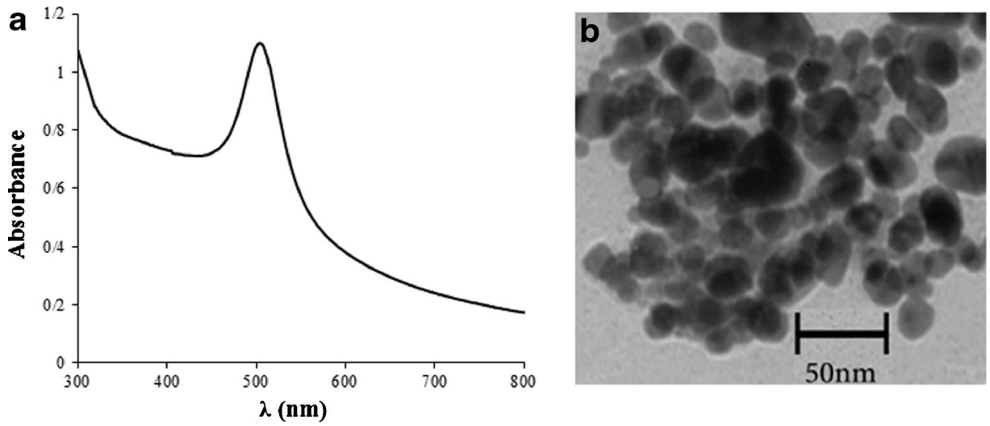


Fig. 2 (**a**) UV–Vis spectra of chitosan- induced Au/Ag alloy NPs, and (**b**) TEM image of chitosan- induced Au/Ag alloy NPs

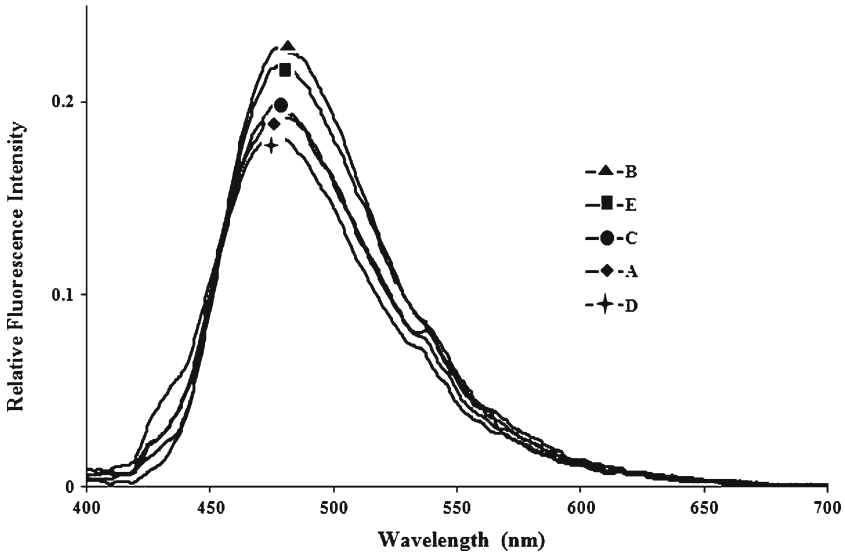


Fig. 3 The fluorescence emission spectra of coumarin derivatives (*A-B-C-D-E*). 5.0×10^{-4} M in methanol, λ_{ex}=385 nm

system. On this basis, the CL reaction must follow a chemically initiated electron exchange luminescence mechanism via the formation of a high energy intermediate(s) such as 3-aminophetalate [57], which is enables the excitation of various fluorophores having emission wavelengths from the ultraviolet to near infrared. The substantial overlapping areas between the absorption spectra of coumarin derivatives (λex≈385 nm) and emission spectrum of luminol (λ_{em}=425 nm) make the process of CRET possible (Fig. 5). The energy of luminol CL excites coumarin derivatives. Because luminol has lower quantum yield whereas coumarins have high quantum yield that nearly reaches 70 %, a great number of coumarin derivatives molecules are excited to emit fluorescence, thus the total CL intensity is dramatically increased [58]. Considering the properties of suitable fluorophores, Π conjugated compounds containing donor substitutes and rigid structures are the best fluorescent brighteners [59]. The sequence of coumarin derivatives effectiveness on the CL intensity of luminol-H_2O_2 system follows these principles and confirms the quality of their effect as fluorophores on the CL system. The presence of tert-butyl in carboxylate group (compound B & E) as a stronger electron-donor substituent in comparison with ethyl (compound C) and methyl (compound A & D) on the side chain of coumarin ring and also, displacement of cyclohexylamine group (compound E & D) by a the tert-butylamine group (compound A, B & C) at the tail end of side chain of coumarin ring increases electron charge of coumarin ring and consequence π conjugation. With increasing π conjugation the energy difference between HOMO and LUMO of coumarin derivatives (as fluorophore) decreases and lead to an increase in the resonance forms; it suggests the feasibility of absorption and fluorescence through CL resonance energy transfer of luminol (donor) to fluorophore (acceptor) and consequently the resonance energy transfer efficiency.

To evaluate the simultaneous effect of chitosan-induced Au/Ag alloy NPs and coumarin derivatives on the CL intensity, NPs were added to H_2O_2-luminol CL system in the presence each of coumarin derivatives. The very strong CL was obtained in the simultaneous presence of them, although order of the coumarin derivatives effect was changed (Fig. 4b).

As mentioned, the presence of chitosan-induced Au/Ag nanoalloy in the CL system, in addition to enhance the CL intensity of fluorophore-H_2O_2-luminol system can alter the effect sequence of fluorophores on the CL system. Whereas,

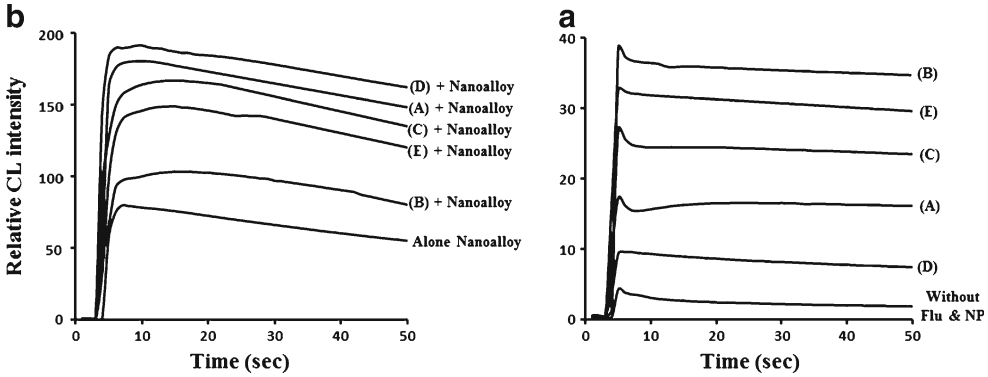


Fig. 4 CL spectra of luminol-H_2O_2 system: (**a**) In presence of fluorophores with chitosan-induced-Au/Ag nanoalloy, (**b**) In presence of fluorophores without chitosan-induced-Au/Ag nanoalloy. Conditions: [luminol] = 1.5×10^{-4} M, [chitosan-induced-Au/Ag nanoalloy] = 4.5×10^{-6} M, [fluorophore] = 1.0×10^{-3} M, [H_2O_2] = 1.0×10^{-4} M, pH = 8.0)

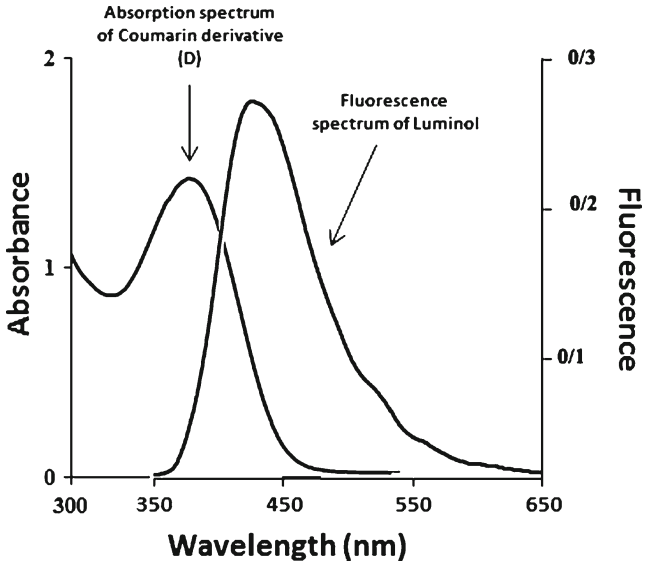


Fig. 5 Absorption spectrum of Coumarin derivative (D) and fluorescence spectrum of Luminol. Conditions: [Coumarin derivative] = 1.0×10^{-3} M, [Luminol] = 1.0×10^{-4} M

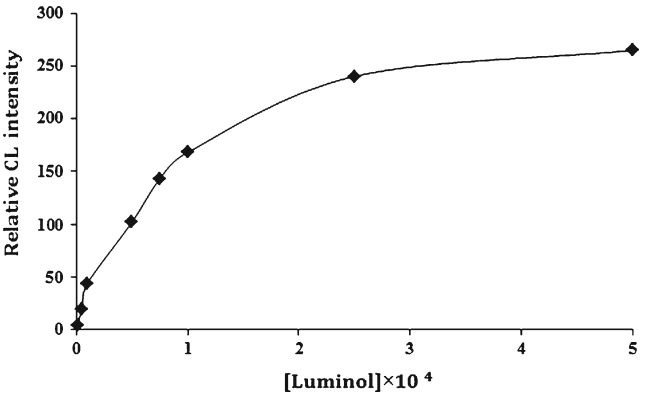


Fig. 7 CL spectra for (*solid line*) luminol-H_2O_2, (*dotted line*) fluorophore-luminol-H_2O_2 and (*dashed line*) fluorophore- Au/Ag nanoalloy-luminol-H_2O_2 systems. Conditions: ([luminol] = 5.0×10^{-4} M, [Au/Ag nanoalloy] = 4.5×10^{-6} M, [fluorophore] = 1.0×10^{-3} M, [H_2O_2] = 1.0×10^{-4} M, pH = 8.0)

the energy transfer process depends on the collision of the molecules of fluorophores and luminol [32]; it seems the probability of this collision can be changed in presence of chitosan-induced Au/Ag nanoalloy.

In fact, chitosan-induced Au/Ag alloy NPs are nanoparticles embedded in chitosan matrix. Metal nanoparticles are a suitable surface for adsorption of compounds such as luminol radical and superoxide anion radical [40, 41]. On the other hand, the spatial arrangement of the chitosan chains on the surrounding NPs makes interaction between active functional groups of chitosan and fluorophore possible. On these bases, it can be concluded that chitosan as a framework including all of CL reagent such as fluorophore, NPs and luminol supplies a suitable platform for CL reaction and can induce increasing of CL intensity due to the increasing rigidity of system. Definitely, whatever interaction between chitosan and fluorophore is stronger, the CL emission is brighter. Steric inherent around the fluorophore's carbonyl group decreases the interaction strength that can prevents fluorophore to take on the exposure of other CL reagents on the framework of chitosan and consequently their efficiency as brightener fluorescent are weakened.

In the following, order of the coumarin derivatives effect on the CL intensity in the presence and absence of NPs are shown:

Coumarin derivatives−luminol−H_2O_2CL system : B > E > C > A > D

Au/Ag nanoalloy−Coumarin derivatives− luminol−H_2O_2CL system : D > A > C > E > B

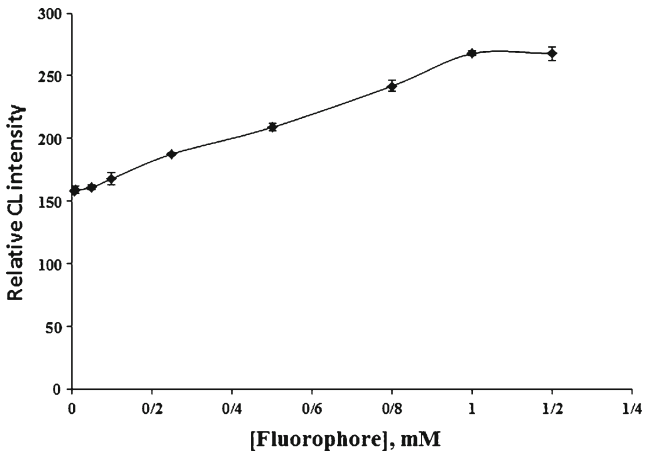


Fig. 6 Effect of fluorophore (D) concentration on the CL intensity of Au/Ag nanoalloy-fluorophore (D)- luminol-H_2O_2 system. Conditions: ([Au/Ag nanoalloy] =, [luminol] = 5.0×10^{-4} M, [H_2O_2] = 1.0×10^{-3} M, pH = 8.0)

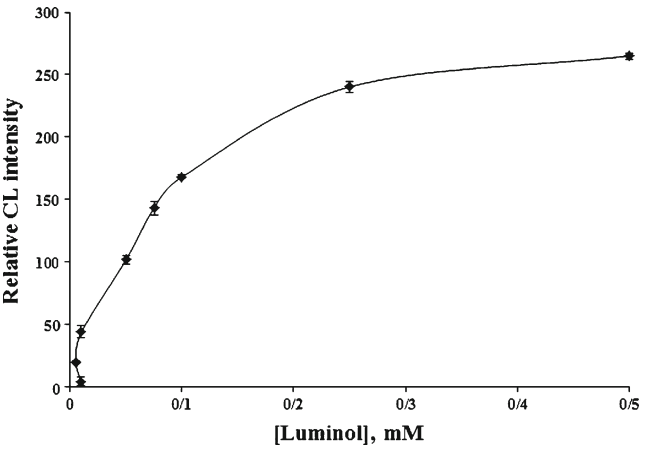


Fig. 8 Effect of luminol concentration on the CL intensity of Au/Ag nanoalloy-fluorophore (D) -luminol-H_2O_2 system. Conditions: ([Au/Ag nanoalloy] = 4.5×10^{-6} M, [fluorophore] = 1.0×10^{-3} M, [H_2O_2] = 1.0×10^{-3} M, pH = 8.0)

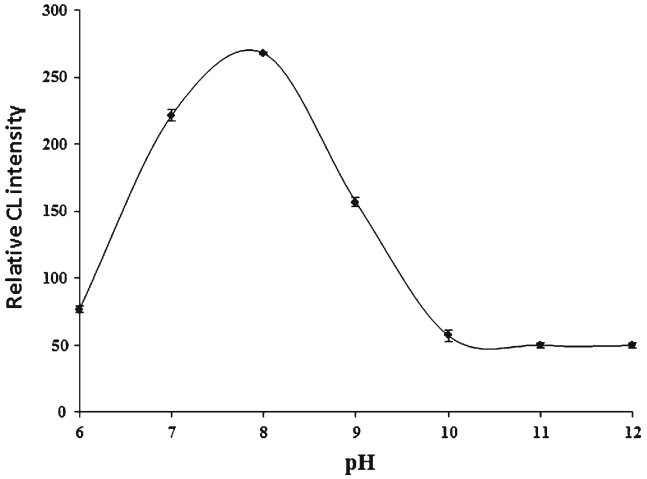


Fig. 9 Effect of pH on the CL intensity of Au/Ag nanoalloy-fluorophore (D)-luminol-H_2O_2 system, Conditions: ([luminol] = 5.0×10^{-4} M, [Au/Ag nanoalloy] = 4.5×10^{-6} M, [fluorophore] = 1.0×10^{-3} M, [H_2O_2] = 1.0×10^{-3} M, pH = 8.0)

Optimization of Fluorophore Concentration

According to above findings dimethyl 2-(cyclohexylcarbamoyl)-3-(4-(trifluoromethyl)-2-oxo-2H-chromen-7-ylamino) succinate (D) was determined as the most efficient fluorescent molecule on this CL system and further studies were performed on it.

The concentration influence of the most efficient fluorophore on the CL intensity of fluorophore-Au/Ag nanoalloy-luminol-H_2O_2 system was studied at constant concentrations of other reagents. As it is clearly shown in Fig. 6, there is an increase in chemiluminescence intensity with increasing concentration of the Fluorophore up to 1.0×10^{-3} M.

Effect of the Concentration of Chitosan-Induced Au/Ag Alloy Nanoparticles

The effect of the NP concentration was investigated in the range of 1.5×10^{-6} to 1.5×10^{-5} M. The CL intensity was increased steadily with increasing concentration of nanoalloy until 4.5×10^{-6} M and then was decreased probably due to the increasing acidic nature of solution. Thus 4.5×10^{-6} M was selected as optimum concentration. Figure 7 shows Au/Ag nanoalloy and fluorophore (D) effects on the CL intensity of luminol-H_2O_2 system.

Optimization of Luminol Concentration

As the luminescence reagent in this system, luminol concentration effects on the CL response. The effect of luminol concentration in the range of 1.0×10^{-6} to 5.0×10^{-4} M was investigated (Fig. 8). The result showed that increasing luminol concentration is followed by a steady increase in CL intensity. However, 5.0×10^{-4} 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 obtained blank signal.

Optimization of pH

In Au/Ag alloy NP-fluorophore (D)-luminol-H_2O_2 CL system, phosphate buffer solution is medium of the CL reaction. There are two factors that determine the overall response of CL to pH: the effect of pH on the generated CL signal, and the influence of pH on interaction between fluorophores and

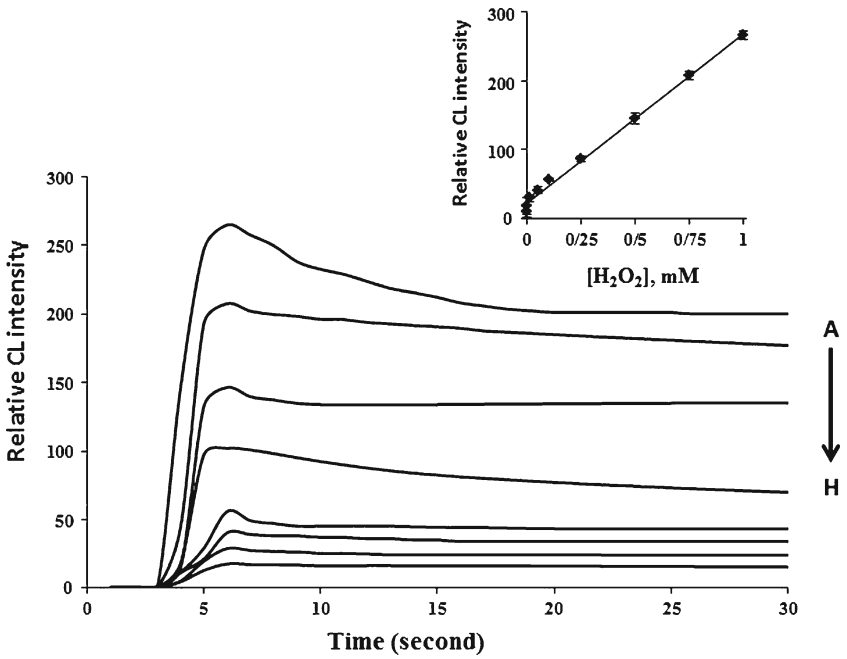


Fig. 10 Calibration curve for glucose determination with Au/Ag nanoalloy-fluorophore (D)-luminol-H_2O_2 system. Conditions: ([luminol] = 5.0×10^{-4} M, [Au/Ag nanoalloy] = 4.5×10^{-6} M, [fluorophore] = 1.0×10^{-3} M

chitosan. In view of the nature of Au/Ag alloy NP catalyzed-luminol CL reaction, which is more favored under basic conditions (pH 10–11) [38], an alkaline medium would improve the sensitivity of the system. Furthermore, acidic pH can debilitate fluorescence strength of coumarin derivatives as fluorophore, it is related to protonation of NH group in 7-position which decreases resonance stretches. On the other hand, the optimal pH value for interaction active groups of chitosan with fluorophores is 5 [60]. So, the general effect of medium final pH on the CL response was investigated (Fig. 9). The CL intensity reached the highest value at pH 8.0. As such, the reaction was performed at pH 8.0 throughout this study.

Hydrogen Peroxide Detection

Under optimized conditions, as shown in Fig. 10, the CL intensity of Au/Ag alloy NP-fluorophore (D)-luminol-H_2O_2 system was linear with the H_2O_2 concentration in the range of 1.0×10^{-7}–1×10^{-3} M, with the correlation coefficient of 0.996. The limit of detection ($3\sigma/b$) was 6.0×10^{-8} M. Such performance of the hydrogen peroxide detection is comparable with the best published results using electrochemical biosensor [61–64] or luminescence methods [65, 66].

Sample Analysis

The linear response range of 1.5×10^{-6}–5.0×10^{-3} M and the detection limit of 7.5×10^{-7} M was found for the glucose standards (Fig. 11). To investigate the feasibility of the sensing system for glucose analysis in biological samples, glucose concentration in human serum and urine were examined. The samples were collected from medical diagnostic laboratory and used as testing samples. The fresh samples were first analyzed with a BT3000 Biochemical Analyzer, which used spectrophotometric method combined with the enzymatic reaction of GOD and HRP [61].

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

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	235.3±1.3	232	13.7±0.5	6
2	127.5±3.0	129	4.5±0.1	–
3	54.8±2.1	54	2.8±0.4	–

[a] LOD of the Pars Azmoon photometric kit is 5 mg dL^{-1}

Possible CL Mechanism

The CL mechanism of the luminol–H_2O_2 reaction has been discussed by Merényi and Burdo [67, 68]. They confirmed that a highly energy excited intermediate of 3-aminophthalate was the emitter in the luminol– H_2O_2 CL

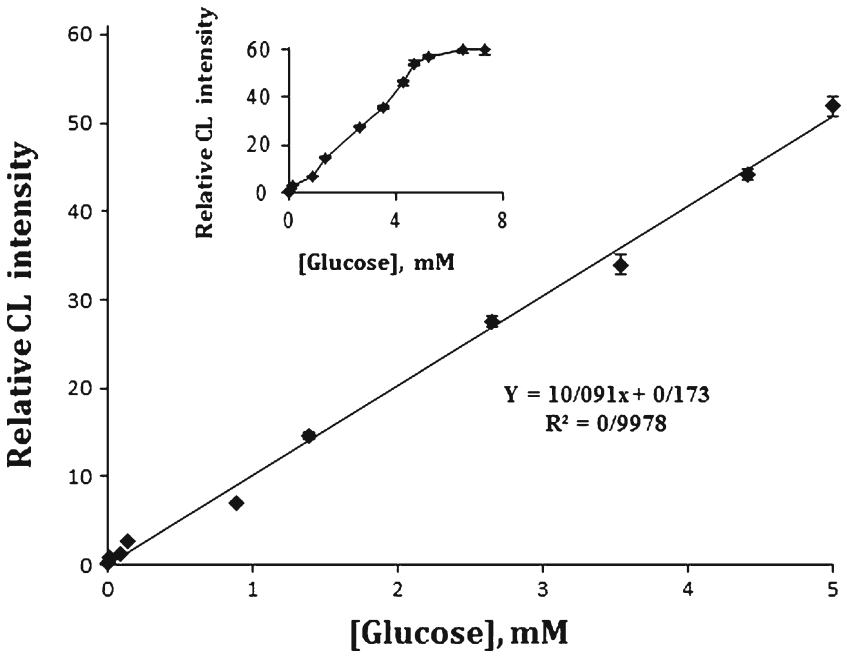


Fig. 11 Chemiluminescence emission intensity as a function of time for Au/Ag nanoalloy-fluorophore(D)-luminol-H_2O_2 system with constant concentration of luminol, 5.0×10^{-4} M; fluorophore, 1.0×10^{-3} M; Au/Ag nanoalloy, 4.5×10^{-6} M and varying concentrations of H_2O_2: (A) 1.0×10^{-3}, (B) 7.5×10^{-4}, (C) 5.0×10^{-4}, (D) 2.5×10^{-4}, (E) 1.0×10^{-4} M, (F) 5.0×10^{-5} M, (G) 1.0×10^{-5} M and (H) 1.0×10^{-6} M. Corner of right hand side: The correlation diagram for the chemiluminescence emission with H_2O_2 concentrations

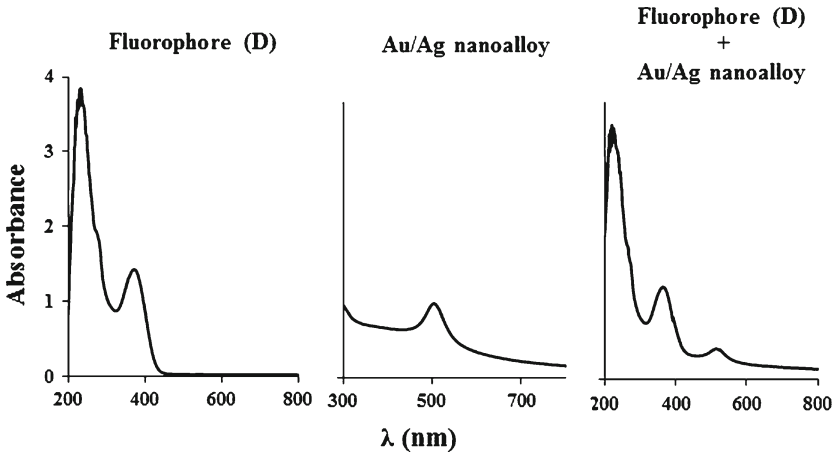


Fig. 12 UV–Vis spectra of fluorophore (D), Au/Ag nanoalloy, and their mixture. Conditions: ([Au/Ag nanoalloy] = 4.5×10^{-6} M, [fluorophore] = 1.0×10^{-3} M and volume ration of Flu to NP = 10)

Scheme 2 Summary of possible CL reaction mechanism

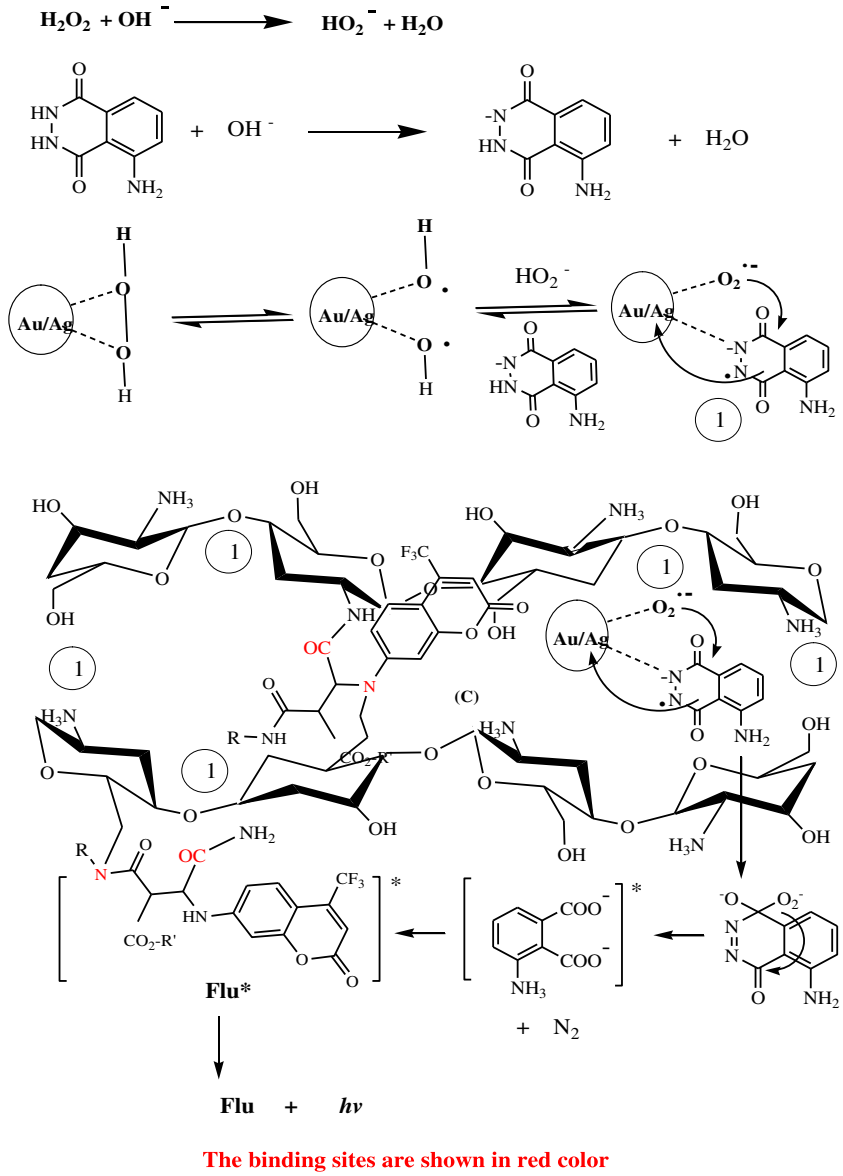


Table 2 Tolerance folds coexisting substances

Tolerance folds	Coexisting substances
≥1000	Na^+, K^+, Ca^{2+}, Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, starch
≥100	Thiourea, urea lactic acid, uric acid, and citric acid
≥50	Bilirubin, sucrose, maltose, fructose and lactose
≥10	Ascorbic acid

reaction [69]. In the presence of an efficient fluorophore such as coumarin derivative, the excited intermediate of 3-aminophthalate can be involving the chemiexcitation of a fluorophore, via conversion of the chemical energy into electronic excitation energy or in other word, resonance energy transfer. At the end, the excited fluorophore molecule returns to the ground state and liberating "light [32].

Coumarin derivatives are simply applied as the energy acceptor and do not join the CL reaction. The presence of coumarin derivatives as fluorophore does not affect the detector but the enhanced CL makes the system collect stronger signal and the analytes can be analyzed more precisely and easily.

During oxidation of luminol oxygen-related radicals (for example, $OH^{\cdot}$, $O_2^{\cdot -}$, and others), as oxidants, are expected to be generated from H_2O_2 [69]. Previous findings about Au NPs catalyzed-luminol CL reaction revealed that the oxidation state of Au was not involved in catalysis of the CL process; the origin of the CL was therefore assigned to Au nanoparticles themselves. According to Zhang [36], when metal nanoparticles are added to the luminol– H_2O_2 CL system, H_2O_2 might be absorbed on the surface of the metal nanoparticles, causing partial electron transfer from the metal nanoparticles to the adsorbed H_2O_2, and the O–O bond of H_2O_2 might be broken into two $OH^{\cdot}$ radicals which react with luminol and HO_2^- to facilitate formation of luminol radicals and superoxide radical anion ($O_2^{\cdot -}$) on the surface of the nanoparticles, leading to enhancement of CL intensity.

As mentioned in Result and Discussion section, in addition to catalysis CL reaction, chitosan-induced Au/Ag NPs can make a difference to the process of the fluorophores effect on the fluorophore-luminol–H_2O_2 CL system.

UV–Vis absorption spectra of fluorophore (D), Au/Ag nanoalloy, and their mixture are shown in Fig. 12. It can be seen that NP has a notable absorption peak at approximately 510 nm and fluorophore (D) has two distinct absorption peaks at approximately 230 and 385 nm. When fluorophore is mixed with NPs no new absorption peaks appeared, and the absorption spectrum of the mixed system is approximately the sum of two absorption spectra; with slight change in absorption spectrum fluorophore. Hence, alteration the process of fluorophores effect seems be due to the chitosan properties. Chitosan chains on the surrounding NPs can interact with carbonyl and amino groups of fluorophore (The bindings sites are shown in red color). However, the increasing of spatial inhibition around fluorophore's carbonyl groups reduces the interaction. Moreover, as mentioned the embedded Au/Ag nanoalloys in chitosan matrix are a suitable surface for placement of compounds such as luminol radicals and $O_2^{\cdot -}$ radicals. On these bases, chitosan like a framework containing fluorophore, NPs, luminol radicals and $O_2^{\cdot -}$ radicals provides a suitable platform for CL reaction that electron transfer process can take on it and it induces a strong CL due to rigidity system. Scheme 2. shows a summary of possible CL reaction mechanism.

Interference Studies

The effects of various interfering species which may accompany glucose in human serum and urine were studied. Glucose (1×10^{-5} M) was placed in the reaction cell and increasing amounts of interfering substances were added into the solution. All species tested were tolerated at reasonably high concentrations; confirm the high selectivity of the proposed method. It is worth to mention that ascorbic acid at high concentration can influence on the 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 system response to glucose. 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.

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

Method	Linear range (M)	Detection limit (M)	Reference
New Fluorophore- peroxyoxalate chemiluminescence	2.50×10^{-6}–1.75×10^{-4}	1.1×10^{-6}	[22]
Gallium hexacyanoferratemodified carbon ionic liquid paste electrode	1.0×10^{-4}–6.0×10^{-3}	3.0×10^{-5}	[70]
Gold nanosphere –ECL biosensor	1.0×10^{-6}–4.3×10^{-3}	3.0×10^{-7}	[71]
Glucose oxidase/carbon-nanotube /NP in nafion film-CL biosensor	2.25×10^{-6}–1.75×10^{-4}	1.0×10^{-6}	[72]
Boronic anthraquinone derivatives/GOD- fluorescence	8.0×10^{-5}–$4.\ 2\times10^{-4}$	1.1×10^{-5}	[73]
Photopolymerized fluorescence sensor	2.8×10^{-5}–$5.\ 5\times10^{-3}$	0.89×10^{-5}	[74]
This work	1.5×10^{-6}–5.0×10^{-3}	7.5×10^{-7}	–

Conclusion

In this study, simultaneous introducing of chitosan-induced Au/Ag nanoalloy and synthetic fluorophores on the luminol-H_2O_2 CL system provided accessibility to a very low detection limit in determination of glucose based on the CL detection of enzymatically generated H_2O_2. The applied chitosan as stabilizer and reductant in synthesis of Au/Au nanoalloy can interacts with fluorophores' carbonyl groups due to virtue of its abundant groups. Since the embedded Au/Ag nanoalloys in chitosan matrix are a suitable surface for adsorption of luminol radical and superoxide anion radical, chitosan can acts as a platform for all of effective reagents on the CL reaction that induce an enhancement CL in relation to rigidity system. The results demonstrate that the suitable fluorophore and NPs are powerful tools to enlarge CL signals as the sensitivity of present chemiluminescence method permits glucose analysis in the real sample with concentrations as small as 2 mg/dl. A comparison of the main features of the present method with others reported in the literature is given in Table 3.

Acknowledgments The authors thank goes to Danesh clinical laboratory in Babol which provided us serum samples and valuable information.

References

1. Peng J, Wang Y, Wang J, Zhou X, Liu Z (2011) A new biosensor for glucose determination in serum based on up-converting fluorescence resonance energy transfer. Biosens Bioelectron 28:414–420
2. Li J, Yu J, Zhao F, Zeng B (2007) Direct electrochemistry of glucose oxidase entrapped in nano gold particles-ionic liquid-N, Ndimethylformamide composite film on glassy carbon electrode and glucose sensing. Anal Chim Acta 587:33–40
3. Zhao S, Zhang K, Bai Y, Yang W, Sun C (2006) Glucose oxidase/colloidal gold nanoparticles immobilized in Nafion film on glassy carbon electrode: direct electron transfer and electrocatalysis. Bioelectrochemistry 69:158–163
4. Jiang L, Liu H, Liu J, Yang Q, Cai X (2008) A sensitive biosensor based on Os-complex mediator and glucose oxidase for low concentration glucose determination. J Electroanal Chem 619–620:11–16
5. Hecht HJ, Kalisz HM, Hendle J, Schmid RD, Schomburg D (1993) Crystal structure of glucose oxidase from aspergillus niger refined at 2 · 3 Å resolution. J Mol Biol 229:153–172
6. Clark LC, Lyons JC (1962) Electrode systems for continuous monitoring in cardiovascular surgery. Ann N Y Acad Sci 102:29–45
7. Updike SJ, Hikcks GP (1967) The enzyme electrode. Nature 214: 986–988
8. Cass AEG, Davis G, Francis GD, Hill HAO, Aston WJ, Higgins J, Scott LDL, Plotkin EV, Turner APF (1984) Ferrocene-mediated enzyme electrode for amperometric determination of glucose. Anal Chem 56:667–671
9. DCCT Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 329:977–986
10. Keston AS (1956) Specific colorimetric enzymatic analytical reagents for glucose. Abstract of Papers, 129th Meeting, ACS, Dallas, Texas, p. 31C
11. Matsubara C, Kudo K, Kawashita T, Takamura K (1985) Spectrophotometric determination of hydrogen peroxide with titanium 2-((5-bromopyridyl)azo)-5-(N-Pro-pyl-N sulfopropylamino)phenol reagent and its application to the determination of serum glucose using glucose oxidase. Anal Chem 57:1107–1109
12. Chen X, Yan X, Khor KA, Tay BK (2007) Multilayer assembly of positively charged polyelectrolyte and negatively charged glucose oxidase on a 3D Nafion network for sensoring glucose. Biosens Bioelectron 22:3256–3260
13. Yao T, Takashima K (1998) Amperometric biosensor with a composite membrane of sol–gel derived enzyme film and electrochemically generated poly (1,2-diaminobenzene) film. Biosens Bioelectron 13: 67–73
14. Wang B, Li B, Deng Q, Dong S (1998) Amperometric glucose biosensor based on sol–gel organic–inorganic hybrid material. Anal Chem 70:3170–3174
15. Wang J, Musameh M, Lin Y (2003) Solubilization of carbon nanotubes by Nafion toward the preparation of amperometric biosensors. J Am Chem Soc 125:2408–2409
16. Zhang S, Wang N, Niu Y, Sun C (2005) Immobilization of glucose oxidase on gold nanoparticles modified Au electrode for the construction of biosensor. Sensors Actuators B Chem 109:367–374
17. Reiter S, Habermüller K, Schuhmann W (2001) A reagentless glucose biosensor based on glucose oxidase entrapped into osmium-complex modified polypyrrole films. Sensors Actuators B Chem 79:150–156
18. Luo X, Xu J, Du Y, Chen H (2004) A glucose biosensor based on chitosan–glucose oxidase–gold nanoparticles biocomposite formed by one-step electrodeposition. Anal Biochem 334:284–289
19. Bostick DT, Hercules DM (1975) Quantitative determination of blood glucose using enzyme induced chemiluminescence of luminol. Anal Chem 47:447–452
20. Economou A, Panoutsou P, Themelis DG (2006) Enzymatic chemiluminescent assay of glucose by sequential-injection analysis with soluble enzyme and on-line sample dilution. Anal Chim Acta 572: 140–147
21. Kanchana W, Sakai T, Teshima N, Katoh S, Grudpan K (2007) Successive determination of urinary protein and glucose using spectrophotometric sequential injection method. Anal Chim Acta 604: 139–146
22. Zargoosh K, Shamsipur M, Qandalee M, Piltan M, Moradi L (2011) Sensitive and selective determination of glucose in human serum and urine based on the peroxyoxalate chemiluminescence reaction of a new Fluorophore. Spectrochim Acta A Mol Biomol Spectrosc 81: 679–683
23. Matsumoto K, Waki K (1999) Simultaneous biosensing of ethanol and glucose with combined use of a rotating bioreactor and a stationary column reactor. Anal Chim Acta 380:1–6
24. Li B, Zhang Z, Jin Y (2001) Chemiluminescence flow sensor for in vivo on-line monitoring of glucose in awake rabbit by microdialysis sampling. Anal Chim Acta 432:95–100
25. Fang Q, Shi XT, Sun YQ, Fang ZL (1997) A flow injection microdialysis sampling chemiluminescence system for in vivo on-line monitoring of glucose in intravenous and subcutaneous tissue fluid microdialysates. Anal Chem 69:3570–3577
26. Obata H, Karatani H, Nakayama E (1993) Automated determination of iron in seawater by chelating resin concentration and chemiluminescence detection. Anal Chem 65:1524–1528
27. Lin QX, Guiraum A, Escobar R (1993) Flow-injection chemiluminescence determination of cobalt(II) and manganese(II). Anal Chim Acta 283:379–385
28. Li ZP, Li KA, Tong SY (1999) Study of the catalytic effect of copper(II)-protein complexes on luminol-H_2O_2 chemiluminescence reaction and its analytical application. Anal Lett 32:901–913

29. Wang XM, Teng XL, Ma YJ, Zhou M, Li L, Chen H (2008) The chemiluminescence spectra of acidic potassium permanganate-galangin system. Chin J Lumin 29:583–585
30. Ci XY, Chang WB, He HB, Yue Z (1989) The quenching of o-phenanthroline fluorescence by nucleic acids and its analytical applications. Chin J Anal Lab 8:15–17
31. Cherrine KP, Angel MR, Miguel DLG, José LFCL, Elias AGZ, Boaventura FR (2007) A chemiluminescence flow-based procedure for determination of carbaryl in natural waters exploiting multicommutation and enzymatic reaction. J Braz Chem Soc 18: 519–525
32. Xu H, Liu CM, He Y, Tang HW, Wu QS (2010) Study on the chemiluminescence resonance energy transfer between luminol and fluorescent dyes using a linear CCD spectrometer. J Lumin 130: 1872–1879
33. Ciscato LF, Bartoloni FH, Weiss D, Beckert R, Baader WJ (2010) Experimental evidence of the occurrence of intramolecular electron transfer in catalyzed 1,2-dioxetane decomposition. J Org Chem 75: 6574–6580
34. Li Z, Wang Y, Zhang G, Xu W, Han Y (2010) Chemiluminescence resonance energy transfer in the luminolCdTe quantum dots conjugates. J Lumin 130:995–999
35. Chen W, Hong L, Liu AL, Liu JQ, Lin XH, Xia XH (2012) Enhanced chemiluminescence of the luminol-hydrogen peroxide system by colloidal cupric oxide nanoparticles as peroxidase mimic. Talanta 99: 643–648
36. Zhang ZF, Cui H, Lai CZ, Liu LJ (2005) Gold nanoparticle-catalyzed luminol chemiluminescence and its analytical applications. Anal Chem 77:3324–3329
37. Duan C, Cui H, Zhang Z, Liu B, Guo J, Wang W (2007) Size-dependant inhibition and enhancement by gold nanoparticles of luminol-ferricyanide chemiluminescence. J Phys Chem C 111: 4561–4566
38. Cui H, Guo JZ, Li N, Liu LJ (2008) Gold nanoparticle triggered chemiluminescence between luminol and $AgNO_3$. J Phys Chem C 112:11319–11323
39. Safavi A, Absalan G, Bamdad F (2008) Effect of gold nanoparticle as a novel nanocatalyst on luminol-hydrazine chemiluminescence system and its analytical application. Anal Chim Acta 610:243–248
40. Chen H, Gao F, He R, Cui DX (2007) Chemiluminescence of luminol catalyzed by silver nanoparticles. J Colloid Interface Sci 315: 158–163
41. Xu SL, Cui H (2007) Luminol chemiluminescence catalysed by colloidal platinum nanoparticles. Luminescence 22:77–87
42. Li S, Tao S, Wang F, Hong J, Wei X (2010) Chemiluminescence reactions of luminol system catalyzed by nanoparticles of a gold/silver alloy. Microchim Acta 169:73–78
43. Lynch I, Dawson KA (2008) Protein-nanoparticle interactions. Rev Nanotoday 3:40–47
44. Lan D, Li B, Zhang Z (2008) Chemiluminescence flow biosensor for glucose based on gold nanoparticle-enhanced activities of glucose oxidase and horseradish peroxidase. Biosens Bioelectron 24: 934–938
45. Kim K, Kim KL, Lee SJ (2005) Surface enrichment of Ag atoms in Au/Ag alloy nanoparticles revealed by surface enhanced Raman scattering spectroscopy. Chem Phys Lett 403:77–82
46. Li F, Chen W, Tang C, Zhang S (2009) Development of hydrogen peroxide biosensor based on in situ covalent immobilization of horseradish peroxidase by one-pot polysaccharide-incorporated sol–gel process. Talanta 77:1304–1308
47. Ngah W, Teong L, Hanafah M (2011) Adsorption of dyes and heavy metal ions by chitosan composites. Rev Carbohydr Polym 83: 1446–1456
48. Fan C, Li W, Zhao S, Chen J, Li X (2008) Efficient one pot synthesis of chitosan-induced gold nanoparticles by microwave irradiation. Mater Lett 62:3518–3520
49. Hao C, Ding L, Zhang XJ, Ju HX (2008) Biocompatible conductive architecture of carbon nanofiber-doped chitosan prepared with controllable electrollable electrodeposition for cytosensing. Anal Chem 79:4442–4447
50. Huang HZ, Yang XR (2003) Chitosan mediated assembly of gold nanoparticles multilayer. Colloids Surf A 226:77–86
51. Lin JH, Qu W, Zhang SS (2007) Disposable biosensor based on enzyme immobilized on Au–chitosan-modified indium tin oxide electrode with flow injection amperometric analysis. Anal Biochem 360:288–293
52. Hua LJ, Hui Z, ShuSheng Z (2009) New bienzymatic strategy for glucose determination by immobilized-gold nanoparticle-enhanced chemiluminescence. Sci China Ser B 52:196–202
53. Still WC, Kahn M, Mitra A (1978) Rapid chromatographic technique for preparative separations with moderate resolution. J Org Chem 43: 2923–2925
54. Brondania D, Scheeren CW, Dupont J, Vieira IC (2009) Biosensor based on platinum nanoparticles dispersed in ionic liquid and laccase for determination of adrenaline. Sensors Actuators B Chem 140:252–259
55. Chaichi MJ, Alijanpour SO (2013) Determination of vitamin C in drugs using of an optimized novel TCPO–Amplex red–gold/silver alloy nanoparticles–H_2O_2 chemiluminescence method by the Box–Behnken design. J Lumin 134:195–200
56. Harish S, Sabarinathan R, Joseph J, Phani KLN (2011) Role of pH in the synthesis of 3-aminopropyl trimethoxysilane stabilized colloidal gold/silver and their alloy sols and their application to catalysis. Mater Chem Phys 127:203–207
57. Schuster GB (1979) Chemiluminescence of organic peroxides. Acc Chem Res 12:366–373
58. Rurack K, Spieles M (2011) Fluorescence quantum yields of a series of red and near-infrared dyes emitting at 600–1000 nm. Anal Chem 83:1232–1242
59. Lakowicz JR (1999) Principle of fluorescence spectroscopy, 2nd edn. Kluwer Academic/Plenum Publishers, NewYork
60. Vauthier C, Zandanel C, Ramon AL (2013) Chitosan-based nanoparticles for in vivo delivery of interfering agents including siRNA. Curr Opin Colloid Interface Sci 18:406–418
61. Yanxia X, Chengguo H, Shengshui H (2008) A hydrogen peroxide biosensor based on direct electrochemistry of hemoglobin in Hb–Ag sol films. Sensors Actuators B Chem 130:816–822
62. Liping M, Ruo Y, Yaqin C, Shihong C (2009) Amperometric hydrogen peroxide biosensor based on the immobilization of HRP on DNA–silver nanohybrids and PDDA-protected gold nanoparticles. J Mol Catal B Enzym 56:215–220
63. Balamurugan A, Chen SM (2009) Silver nanograins incorporated PEDOT modified electrode for electrocatalytic sensing of hydrogen peroxide. Electroanalysis 21:1419–1423
64. Song MJ, Hwang SW, Whang D (2010) Non-enzymatic electrochemical CuO nanoflowers sensor for hydrogen peroxide detection. Talanta 80:1648–1652
65. Santafe AAM, Che BD, Blum LJ, Girard-Egrot AP, Marquette CA (2010) 1-ethyl-3-methylimidazolium ethylsulfate/copper catalyst for the enhancement of glucose chemiluminescent detection: effects on light emission and enzyme activity. Anal Chem 82:2401–2404
66. Li B, Lan D, Zhang Z (2008) Chemiluminescence flow-through biosensor for glucose with eggshell membrane as enzyme immobilization platform. Anal Biochem 374:64–70
67. Merényi G, Lind JS, Eriksen TE (1990) Luminol chemiluminescence: chemistry, excitation, emitter. J Biolumin Chemilumin 5:53–56
68. Burdo TG, Seitz WR (1975) Mechanism of cobalt catalysis of luminol chemiluminescence. Anal Chem 47:1639–1643
69. Li Y, Zhu C, Wang L (2005) A highly sensitive and selective assay for cysteine using the chemiluminescence reaction of luminol and hydrogen peroxide. Microchim Acta 150:95–99

70. Haghighi B, Khosravi M, Barati A (2014) Fabrication of gallium hexacyanoferrate modified carbon ionic liquid paste electrode for sensitive determination of hydrogen peroxide. Mater Sci Eng C Mater 40:204–211
71. Zhong X, Chai YQ, Yuan R (2014) A novel strategy for synthesis of hollow gold nanosphere and its application in electrogenerated chemiluminescence glucose biosensor. Talanta 128:9–14
72. Zargoosh K, Chaichi MJ, Shamsipur M, Asghari S (2012) Highly sensitive glucose biosensor based on the effective immobilization of glucose oxidase/carbon-nanotube and gold nanoparticle in nafion film and peroxyoxalate chemiluminescence reaction of a new fluorophore. Talanta 93:37–43
73. Wannajuk K, Jamkatoke M, Tuntulani T, Tomapatanaget B (2012) Highly specific-glucose fluorescence sensing based on boronic anthraquinone derivatives via the GOx enzymatic reaction. Tetrahedron 68:8899–8904
74. Cubuk S, Yetimoglu EK, Kahraman MV, Demiribilek D, Firlak M (2013) Development of photopolymerized fluorescence sensor for glucose analysis. Sensors Actuators B Chem 181:187–193