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Chemiluminescence determination of timolol maleate by gold nanoparticles-catalyzed luminol–N-bromosuccinimide system

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ABSTRACT

A new chemiluminescence (CL) method combined with flow injection technique was developed for the determination of timolol maleate. Gold nanoparticles was found to catalyze the CL reaction of luminol with N-bromosuccinimide in an alkaline condition. The CL signal was furtherly enhanced significantly when timolol maleate was presented in the reaction system. But timolol maleate alone inhibited the CL signal from luminol–N-bromosuccinimide reaction slightly. Under the selected conditions, the enhanced CL intensity was linearly related to timolol maleate concentration in the range of 0.01–5.0 mg/L with a detection limit of 7.6 μg/L. The relative standard deviation was 2.7% for 11 repeated measurements of 0.1 mg/L timolol maleate solution. The proposed method was applied to the determination of timolol maleate in eye drops and in spiked human urine. A discussion on the possible CL reaction mechanism was also presented.

1. Introduction

Timolol maleate, (+)-3-[3-(tert-butylamino)-2-hydroxy-propoxy]-4-morpholino-1,2,5-thiadiazole monomaleate, is a non-selective β-adrenergic blocker clinically used in the treatment of acute myocardial infarction, angina pectoris, hypertension and glaucoma [1–3]. Various analytical methods have been reported for the determination of timolol maleate in pharmaceutical preparations and in biological fluids, including spectrophotometry [4,5], voltammetry [6,7], chromatography [8,9], capillary electrophoresis [10], and mass spectrometry [11].

Chemiluminescence (CL) method has the advantage of high sensitivity with relatively simple and inexpensive instrumentation. It also offers high analytical throughput when combined with flow injection analysis. Few CL methods were suggested for the determination of timolol maleate, which were based upon the CL reactions of tetrachloroaurate(III)–luminol [12] and soluble Mn(IV)–formaldehyde [13]. The detection limits of 0.5 mg/L [12] and 0.3 mg/L [13] were reported, respectively.

Recently, more and more attentions have been paid to nanomaterials participating in CL systems to improve sensitivity and stability [14]. In these CL systems, nanomaterials participate in the CL reactions as a catalyst, a reductant, a luminophor or an energy acceptor. Gold nanoparticles (AuNPs) is one of the most extensively studied nanomaterials in CL reactions. Previous publications indicated that AuNPs could participate in the CL reactions of luminol with a variety of oxidants, including H_2O_2 [15,16], $K_3Fe(CN)_6$ [17] KIO_4 [18,19], $KMnO_4$ [20], $AgNO_3$ [21] and dissolved O_2 [22].

Halogen-containing oxidants are an important kind of oxidants in the CL reactions [23]. They can oxidize luminol to generate CL in alkaline condition. Reviewing the literature revealed that, up to date, the behavior of AuNPs in the CL reaction of luminol with halogen-containing oxidants still remains unknown.

We here investigated the behavior of AuNPs in the CL reaction of luminol with halogen-containing oxidants using N-bromosuccinimide (for its better stability than hypohalites) as a model oxidant. It was found that AuNPs with the sizes of 6–99 nm had catalyzing effect on the N-bromosuccinimide–luminol CL reaction. The presence of timolol maleate in the reaction system furtherly increased the CL signal significantly. But timolol maleate alone has a slight inhibitory effect on the CL reaction between N-bromosuccinimide and luminol. This reaction system has been developed as a flow injection CL method for the determination of timolol maleate. The experimental conditions for the reaction were carefully optimized and the CL reaction mechanism was thoroughly discussed. The proposed method was applied to the determination of timolol maleate in eye drops and in spiked human urine samples with satisfactory results.

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2. Experimental

2.1. Apparatus

CL measurements were carried out on an IFFM-E flow injection CL analyzer (Xi'an Remax Analytical Instrument Co. Ltd., China). CL spectra were drawn by using an F-4600 spectrofluorimeter (Hitachi Limited, Japan). Absorption spectra were taken on a TU-1901 spectrophotometer (Beijing Currency Instrumental Ltd., China). TEM images of 16 nm AuNPs before and after the CL reaction were measured on a JEM-2100 transmission electron microscope (Japan Electronic Company, Japan).

2.2. Reagents and solution

All chemicals were of analytical grade; deionized distilled water was used throughout. The reagents used were of timolol maleate (Chinese Pharmaceutical and Biological Test Institute, China), luminol (Shaanxi Normal University, China), N-bromosuccinimide (Tianjin Fuchen Chemical Reagents Factory, China), chloroauric acid (Shanghai Chemical Reagent Co. Ltd., China), sodium citrate (Xi'an Chemical Reagent Factory, China), sodium borohydride (Shanghai Sanpu Chemical Industry Co. Ltd., China).

Timolol maleate stock solution (1.0 mg/mL) was prepared by dissolving 0.1000 g of timolol maleate in 100 mL of water. This solution was protected from light and stored at 4 °C in a refrigerator. Working solutions were prepared by gradually diluting the stock solution with water. Luminol solution was prepared by diluting 0.01 mol/L luminol stock solution (prepared in 0.01 mol/L NaOH) to the desired concentration with 0.05 mol/L NaOH solution. Solutions of 1.0 mmol/L N-bromosuccinimide, 0.01% $HAuCl_4$, 1% $Na_3C_6H_5O_7$ and the mixed solution of 0.075% $NaBH_4$/1% $Na_3C_6H_5O_7$ were prepared in water.

2.3. Synthesis of gold nanoparticles

AuNPs with a diameter of 6 nm was synthesized by sodium borohydride reduction method [24]. In brief, 0.40 mL of 1% $Na_3C_6H_5O_7$ solution was added into 100 mL of 0.01% $HAuCl_4$ solution and stirred for 1 min. Then 0.15 mL of 0.075% $NaBH_4$/1% $Na_3C_6H_5O_7$ mixed solution was added and stirred for another 15 min. AuNPs with the diameters of 16 nm, 25 nm, 38 nm, 68 nm and 99 nm were synthesized by sodium citrate reduction method [25,26]. For the synthesis of 16 nm AuNPs, 100 mL of 0.01% $HAuCl_4$ solution was heated to boil. Two mL of 1% $Na_3C_6H_5O_7$ solution was rapidly added into with vigorously stirring $HAuCl_4$ solution and remained at boiling for 15 min. The heating source was then removed and the solution was continuously stirred for another 15 min. The procedures for the synthesis of 25 nm, 38 nm, 68 nm and 99 nm AuNPs were similar to that of 16 nm AuNPs by decreasing the added volumes of 1% $Na_3C_6H_5O_7$ from 2.0 mL to 1.5 mL, 1.0 mL, 0.6 mL and 0.4 mL, respectively. The as-prepared AuNPs solutions were stored in a 4 °C refrigerator.

2.4. Procedures for CL measurement

Flow lines were connected with luminol solution, AuNPs solution, N-bromosuccinimide solution and timolol maleate solution, respectively. Luminol solution (90 μL) was injected into the AuNPs solution with an injection valve and then combined with the merged stream of timolol maleate solution with N-bromosuccinimide solution to produce CL. The concentration of timolol maleate was quantified by the enhanced CL intensity ΔI ($\Delta I = I_s - I_b$), where I_s was the CL signal in the presence of timolol maleate and I_b was the blank signal.

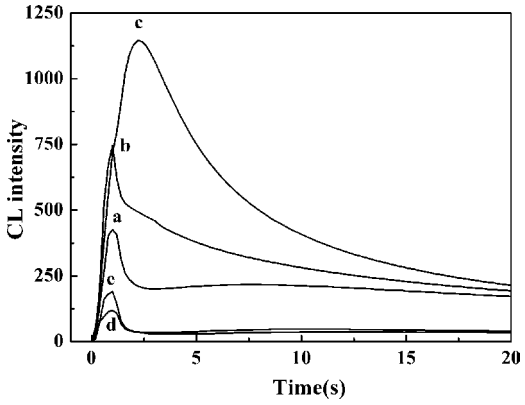


Fig. 1. CL kinetic curves of the injection of 0.50 mL of 40.0 μmol/L N-bromosuccinimide solution into 1.50 mL of 4.0 μmol/L luminol solution (a), the mixture of 4.0 μmol/L luminol–100.0 μmol/L AuNPs (b), 4.0 μmol/L luminol–100.0 μmol/L AuNPs–1.0 mg/L timolol maleate (c), 4.0 μmol/L luminol–1.0 mg/L timolol maleate (d), and 4.0 μmol/L luminol–the supernatant of AuNPs–1.0 mg/L timolol maleate (e).

3. Results and discussion

3.1. CL of timolol maleate in luminol–N-bromosuccinimide reaction catalyzed by AuNPs

The curves a–e in Fig. 1 responds to the injection of 0.50 mL of 40.0 μmol/L N-bromosuccinimide solution into 1.50 mL of 4.0 μmol/L luminol solution, the mixture of 4.0 μmol/L luminol with 100.0 μmol/L AuNPs, 4.0 μmol/L luminol with 100.0 μmol/L AuNPs and 1.0 mg/L timolol maleate, 4.0 μmol/L luminol with 1.0 mg/L timolol maleate, and 4.0 μmol/L luminol with the supernatant of AuNPs and 1.0 mg/L timolol maleate. The CL signal taken from the mixture of 4.0 μmol/L luminol with 100.0 μmol/L AuNPs (curve b) was remarkable higher than that of 4.0 μmol/L luminol solution alone (curve a). Obviously AuNPs displayed a catalyzing effect on the CL reaction of luminol with N-bromosuccinimide. This catalyzing effect could be furtherly increased in the presence of timolol maleate (curve c), which suggested that timolol maleate had an enhancement on the CL signal of luminol–N-bromosuccinimide–AuNPs system. However, in the absence of AuNPs timolol maleate decreased the CL signal from luminol–N-bromosuccinimide reaction slightly (curve d). When AuNPs was replaced by the supernatant of AuNPs (curve e), removing AuNPs by centrifugating AuNPs solution, only a very weak enhanced CL was observed, indicating that the CL caused by the concomitants in the solution could be ignored. Accordingly, the enhancement of Au colloid on the CL was attributed to AuNPs not from other concomitants in the colloid.

3.2. Optimization of experimental conditions

The enhancement of AuNPs on the luminol–N-bromosuccinimide CL reaction was strongly dependent on the sizes and concentrations of AuNPs. As shown in Fig. 2A, all of the examined AuNPs had the enhancement on the CL reaction. The enhanced CL intensity increased with increasing particle size for AuNPs of 6–16 nm. This might be attributed to Fermi level shift, which leads to an alteration of the energy gap for particle-mediated electron-transfer processes [27]. For larger particles (size from 16 to 99 nm diameter) the enhanced CL intensity decreased with increasing particle size, probably because that the particles of smaller size have higher numbers of active sites on their surfaces for a given amount of catalyst material, which lead to a higher rate of catalytic reaction [27]. The enhanced CL intensity increased

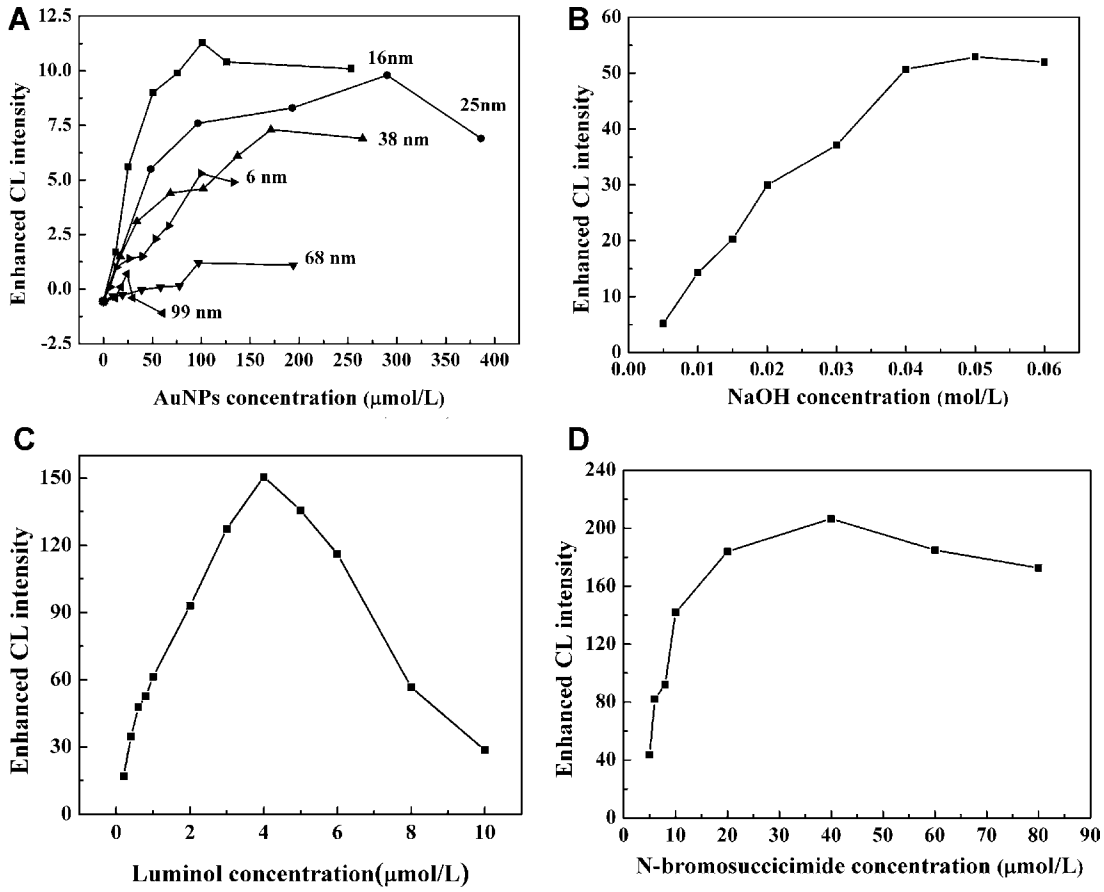


Fig. 2. Effects of reagents concentrations on the enhanced CL intensity. (A) 1.0 μmol/L luminol, 20.0 μmol/L N-bromosuccinimide, 0.01 mol/L NaOH, (B) 1.0 μmol/L luminol, 20.0 μmol/L N-bromosuccinimide, 100.0 μmol/L of 16 nm AuNPs, (C) 20.0 μmol/L N-bromosuccinimide, 0.05 mol/L NaOH, 100.0 μmol/L of 16 nm AuNPs, and (D) 4.0 μmol/L luminol, 0.05 mol/L NaOH, 100.0 μmol/L of 16 nm AuNPs. The concentration of timolol maleate was 0.05 mg/L.

with increase in the concentration of 16 nm AuNPs solution up to 100.0 μmol/L. Above this concentration, the enhanced CL intensity decreased. So, 100.0 μmol/L of 16 nm AuNPs was employed.

Fig. 2B depicts the effect of NaOH concentration on the enhanced CL intensity. In the range of 0.01–0.05 mol/L the enhanced CL intensity increased with NaOH concentration. When the concentration of NaOH was higher than 0.05 mol/L, the enhanced CL intensity decreased. Thus, 0.05 mol/L of NaOH was used.

The effect of luminol concentration on the CL reaction was examined in the range of 0.2–10.0 μmol/L (Fig. 2C). The enhanced CL intensity continued to increase with increase luminol concentration. The enhanced CL intensity reached its maximum at 4.0 μmol/L luminol and this concentration was employed.

As shown in Fig. 2D, the enhanced CL intensity increased with increasing N-bromosuccinimide concentration from 5.0 to 40.0 μmol/L. Higher than 40.0 μmol/L N-bromosuccinimide caused a decrease in the enhanced CL intensity. So, 40.0 μmol/L N-bromosuccinimide was employed.

The effect of flow rate on the enhanced CL intensity was studied over the range of 0.7–2.2 mL/min (each channel). It was observed that the enhanced CL intensity increased with increase in flow rate from 0.7 mL/min to 1.5 mL/min. When the flow rate was above 1.5 mL/min, the enhanced CL intensity decreased probably because of the absence of maximum CL emission in the flow cell. So, the flow rate of 1.5 mL/min was employed.

The effect of the injection volume of luminol solution on the enhanced CL intensity was examined in the range of 50–130 μL. The experiments showed that the enhanced CL intensity increased with increasing the injection volume of luminol solution up to 90 μL. When the injection volume of luminol solution was larger than 90 μL, the enhanced CL intensity decreased. Therefore, 90 μL was employed as the injection volume of luminol solution.

3.3. Performance for timolol maleate measurements

Under the selected experimental conditions, the calibration graph of the enhanced CL intensity (ΔI) vs. timolol maleate concentration (c) was linear in the range of 0.01–5.0 mg/L. The regression equation was $\Delta I = 2.84c(10^{-2}\ \text{mg/L}) + 87.21$ with a correlation coefficient of 0.9972. The relative standard deviation was 2.7% for 11 repeated measurements of 0.1 mg/L timolol maleate solution. The detection limit ($3s_b$) was 7.6 μg/L timolol maleate, which was comparable to that of electrochemical method [6,7] and 1–3 orders of magnitude lower than that of spectrophotometry [4,5], chromatography [8,9], capillary electrophoresis [10], the previously reported CL method [12,13].

3.4. Interference

The effect of foreign species was investigated on the determination of 0.1 mg/L timolol maleate solution. t-Test assumed that, at the confidence level of 95%, no interference has been found when including up to a 1000-fold Na^+, K^+, NO_3^-, Cl^-, SO_4^{2-}; 500-fold Br^-, CO_3^{2-}, urea; 100-fold Ca^{2+}, starch, β-cyclodextrin, citric acid; 50-fold $C_2O_4^{2-}$, tartaric acid; 10-fold Mg^{2+}, Fe^{3+}, PO_4^{3-}, ascorbic acid, glucose, sucrose, lactose, lysine; 5-fold Fe^{2+}, Zn^{2+}, glycine; 1-fold Ba^{2+}, serine, and L-cysteine. However, equal amount of Co^{2+},

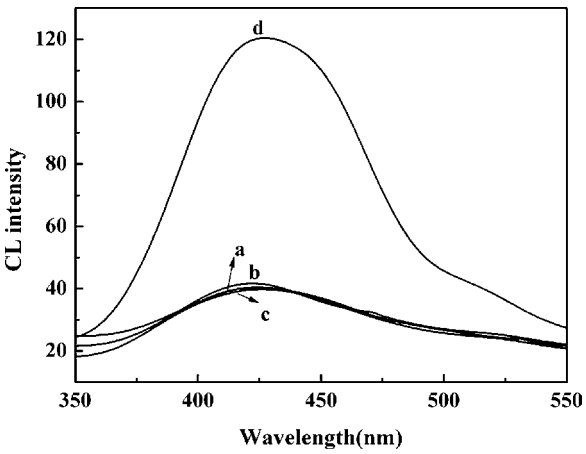


Fig. 3. CL spectra of the reactions of 40.0 μmol/L N-bromosuccinimide–4.0 μmol/L luminol (a), 40.0 μmol/L N-bromosuccinimide–4.0 μmol/L luminol–100.0 μmol/L AuNPs (b), N-bromosuccinimide–4.0 μmol/L luminol–1.0 mg/L timolol maleate (c), and 40.0 μmol/L N-bromosuccinimide–4.0 μmol/L luminol–100.0 μmol/L AuNPs–1.0 mg/L timolol maleate (d).

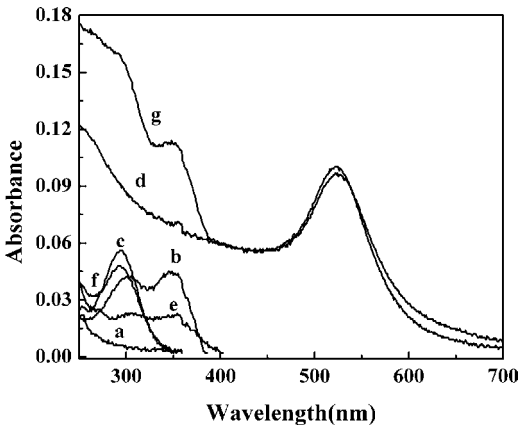


Fig. 4. UV–vis absorption spectra of 10.0 μmol/L N-bromosuccinimide solution (a), 6.3 μmol/L luminol solution (b), 2.5 mg/L timolol maleate solution (c), 25.0 μmol/L AuNPs solution (d), the mixture of 10.0 μmol/L N-bromosuccinimide–6.3 μmol/L luminol (e), 10.0 μmol/L N-bromosuccinimide–2.5 mg/L timolol maleate (f), and 10.0 μmol/L N-bromosuccinimide–6.3 μmol/L luminol–25.0 μmol/L AuNPs–2.5 mg/L timolol maleate (g).

Cu^{2+}, Mn^{2+}, NH_4^+, glutathione, N-acetyl-L-cysteine, and uric acid decreased the CL signal.

3.5. Analytical application

3.5.1. Determination of timolol in eye drops

Commercial eye drops samples containing timolol were purchased from the local market. Without any pretreatment, the eye drops samples was diluted 2×10^4 time with water for analysis. The results were listed in Table 1, *t*-test assumed that there was no significant difference between the results obtained by the proposed method and the Chinese pharmacopoeia method, by measurement of absorbance at 295 nm [28] at the confidence level of 95%.

3.5.2. Determination of timolol maleate in spiked human urine

The urine samples were collected from different healthy volunteers. To 0.20 mL of human urine sample, known amount of timolol maleate standard solution was spiked and mixed. Followed with 1.00 mL of 2% $ZnSO_4$ solution and 1.00 mL of 1.8% $Ba(OH)_2$ solution to remove possible reducing substances in human urine sample [29] and diluted to 4.00 mL with water. After centrifuging at 6000 r/min for 15 min, 0.20 mL of the supernatant was diluted with water to 50 mL for analysis. Blank experiment was also carried out with the same procedure without adding timolol maleate. The results were shown in Table 2. No significant differences had been found between the recoveries and 100% with *t*-test at the confidence level of 95%.

3.6. Discussion on the reaction mechanism

The CL spectra of the reactions were drawn by using an F-4600 spectrofluorimeter in CL mode (turning off excitation source) (Fig. 3). All CL reactions had the similar spectral profile and the same maximum emission wavelength at 425 nm, which suggested that they shared the same CL emitter. The CL emitter of luminol–N-bromosuccinimide reaction was assigned as the excited state of 3-aminophthalate ion [30]. Thus, it was concluded that the excited state of 3-aminophthalate ion was still the CL emitter of the present CL reaction.

As shown in Fig. 4, absorption spectrum taken from 6.3 μmol/L alkaline luminol solution showed two peaks at 346.0 nm and 306.0 nm (curve b). When luminol was mixed with 10.0 μmol/L N-bromosuccinimide, the absorption peaks of luminol at 346.0 nm and 306.0 nm decreased significantly (curve e), which suggested that luminol was oxidized by N-bromosuccinimide in alkaline solution. For absorption spectrum taken from the mixture of 2.5 mg/L timolol maleate with 10.0 μmol/L N-bromosuccinimide, the absorbance peak of timolol maleate at 293.0 nm also decreased obviously (curve f). This result indicated that timolol maleate was also oxidized by N-bromosuccinimide. Compared with

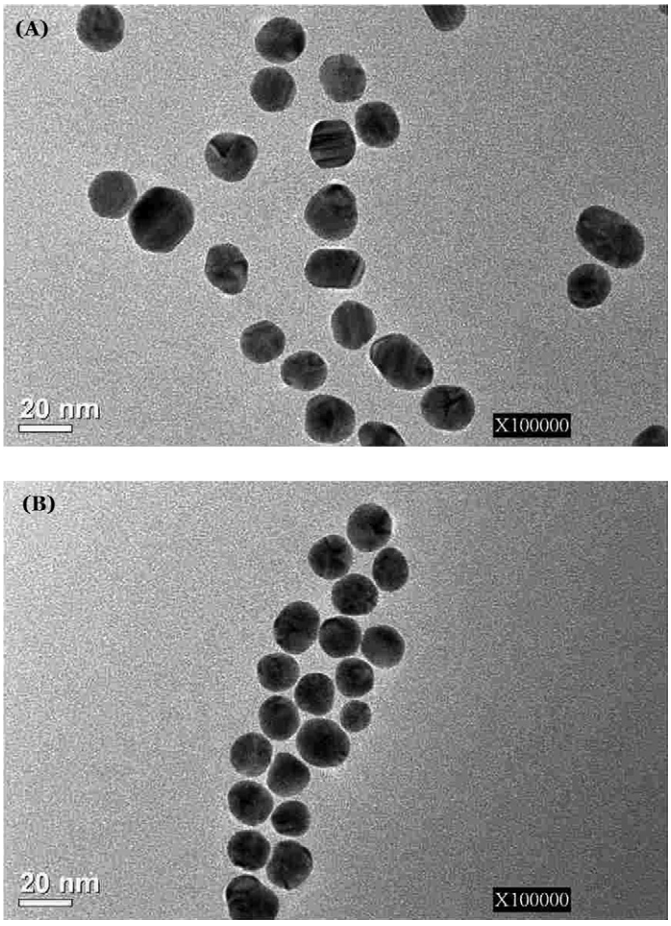


Fig. 5. TEM images of 16 nm AuNPs before (A) and after (B) the CL reaction.

Table 1
Determination of timolol in eye drops.

Samples	Labeled (mg/mL)	This method (mg/mL)	RSD ($n=3$)	Pharmacopoeia method (mg/mL)	RSD ($n=3$)
No. 11030201	5	5.35	1.6%	5.27	0.6%
No. 11020201	5	4.62	2.8%	4.93	0.6%
No. 100601	5	5.03	2.6%	4.95	0.2%

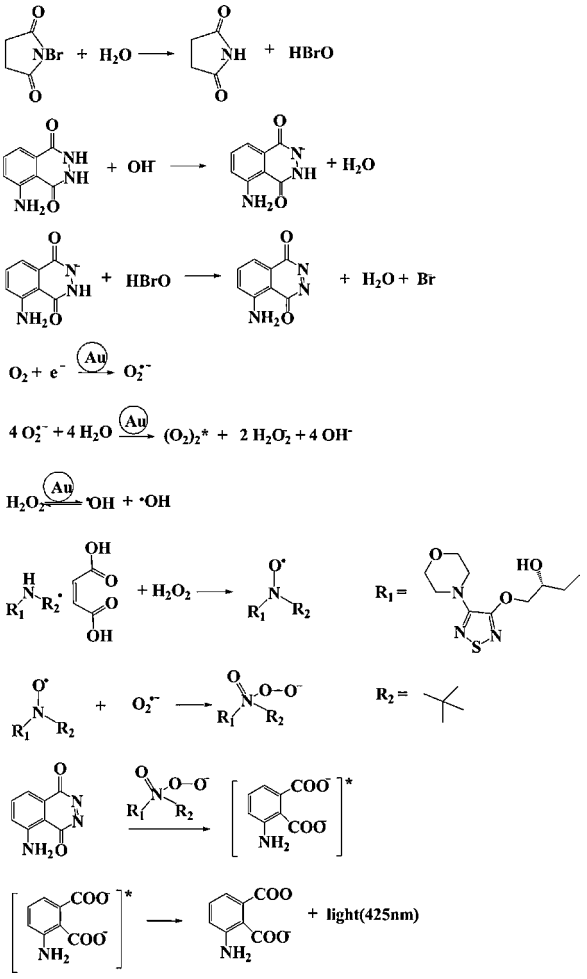
Table 2
Determination of timolol maleate in spiked human urine.

Samples	Added (10^{-2} mg/L)	Found (10^{-2} mg/L)	RSD ($n=3$)	Recovery
No. 1	0.00	0.00	–	–
No. 2	3.00	2.99	3.1%	99.7%
No. 3	5.00	5.18	1.6%	103.6%
No. 4	7.00	6.73	1.8%	96.1%
No. 5	9.00	8.58	1.8%	95.3%

absorption spectrum of 25.0 μmol/L AuNPs solution alone (curve d), the absorption peak of AuNPs at 521.0 nm had no significant differences after the CL reactions (curve g). These results indicated that the structure of AuNPs did not change after the CL reaction, which was consistent with that of TEM images of 16 nm AuNPs before and after the CL reaction (Fig. 5). Thus, it was deduced that AuNPs played a role of a catalyst in the reaction.

When AuNPs was used as the catalyst in reactions, the formation of hydroxyl radical (•OH) and superoxide radical ($O_2^{\bullet-}$) were frequently reported [31–33]. It was reported that the adsorption of oxygen proceeded directly on gold atoms [23] and oxygen vacancies activated oxygen by forming peroxides and superoxides [33,34]. The formed $O_2^{\bullet-}$ further participated in the reactions to generate H_2O_2 under the catalytic action of AuNPs [18]. The generated H_2O_2 adsorbed on the surface of AuNPs. Partial electrons on the surface of AuNPs transferred to the adsorbed H_2O_2, resulting in the broken of O—O band of H_2O_2 into double •OH radicals [15]. These radicals are the important intermediates in luminol CL reaction and can oxidize luminol to generate CL in alkaline condition [35]. To examine whether these oxygen radicals participating in the CL reaction, the effect of scavengers was tested. Superoxide dismutase (SOD, 10.0 mg/L), an effectively scavenger for $O_2^{\bullet-}$, inhibited 46.3% of the CL signal. The addition of •OH scavengers, methanol (10%) and mannitol (0.073 g/mL) [36] into the reaction system inhibited 95.2% and 55.5% of the CL signal, respectively. The CL signal was completely inhibited by common oxygen radical scavengers of ascorbic acid (5.0 mg/L) and thiourea (2.7 mg/mL) [37]. Therefore, it was concluded that these oxygen radicals participated in the CL reaction.

Based on above discussion, the possible CL reaction mechanism was suggested as: the dissolved oxygen is reduced to •OH and $O_2^{\bullet-}$ under the catalytic action of AuNPs. The produced •OH and $O_2^{\bullet-}$ react with luminol in alkaline condition to emit stronger CL. In the absence of AuNPs, timolol maleate reacts with N-bromosuccinimide and reduces the amount of N-bromosuccinimide, causing a slight decrease in CL signal of luminol–N-bromosuccinimide reaction. In the presence of AuNPs, the secondary amine in the molecular structure of timolol maleate reacts with the generated H_2O_2 to form nitroxide radical (NO•) [38]. The formed NO• furtherly reacts with $O_2^{\bullet-}$ to form peroxynitrite radical $ONOO^-$ [39], the latter oxidizes luminol to generate stronger CL [40]. The suggested CL reaction mechanism was shown in Scheme 1.



Scheme 1. Suggested CL reaction mechanism.

4. Conclusion

The CL behavior of AuNPs in luminol–N-bromosuccinimide reaction was explored. AuNPs with the sizes in the range of 6–99 nm catalyzed the CL reaction of luminol with N-bromosuccinimide. A flow injection CL method was developed for the determination of timolol maleate by its enhancing effect on AuNPs-catalyzed luminol–N-bromosuccinimide reaction. The proposed method is simple, sensitive, and has been successfully applied to the determination of timolol maleate in eye drops and in spiked human urine. A possible CL reaction mechanism was also proposed by the studies of CL spectra, UV–vis absorption spectra, TEM images of AuNPs before and after the CL reaction and the effect of scavengers on oxygen-containing radicals.

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