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Gold nanoparticle coupled with fluorophore for ultrasensitive detection of protamine and heparin

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ABSTRACT

Here we report a novel label-free fluorescent sensor for ultrasensitive detection of protamine and heparin based on the high quenching ability of gold nanoparticles to the fluorescence of fluorescein. The fluorescence was significantly quenched when fluorescein molecules were attached to the surface of gold nanoparticles by electrostatic interaction. Upon addition of protamine, the fluorescein molecules were detached from the surface of the gold nanoparticles due to the stronger adsorption of protamine on the surface of AuNPs, and resulting in the recovery of the fluorescein molecules fluorescence. Heparin is able to bind with Protamine specifically. In the presence of heparin, the interaction of heparin with protamine makes the AuNPs de-aggregate and the fluorescein molecules re-attach to the AuNPs, which lead to marked fluorescence quench again. By measuring the changes in the fluorescence of the fluorescein molecules, the concentration of protamine and heparin were sequentially determined. The linear response range was obtained over the concentration range from 0 to 0.8 μg/mL and 4 to 1.6 μg/mL with the low detection limit 0.0067 μg/mL and 0.0013 μg/mL for protamine and heparin, respectively.

1. Introduction

Heparin and protamine are two important polyionic drugs which are widely used in surgical procedures [1]. Heparin, a highly sulfated polyanionic carbohydrate [2], has an average of 70 negative charges per one molecule [3]. It is commercially used as an anticoagulant in surgical procedures for the prevention of blood clotting [1,4]. However, overdose and prolonged use of heparin often induce potentially fatal bleeding complication [1]. Protamine is a highly positively protein with molecular weights of approximately 6 kDa, and an isoelectric point of pH 13.8 [1,3]. It can bind to heparin specifically by electrostatic attraction to form a stable ion pair complex. In order to overcome the risk of heparin-induced bleeding, protamine, the antidote for heparin, is usually administered following surgery to reverse the anticoagulant activity of heparin [1]. Protamine is used as heparin antagonists. In the last few years, protamine has became a promising agent in gene therapy to enhance the lipoplex-mediated gene transfection [5,6] and the delivery of anti-sense oligonucleotides combined with nanoparticles [7,8]. Therefore, the development of sensitive and reliable detection methods with easy operation to monitor heparin and protamine accurately is highly demanded.

Recently, various methods have been established for the determination of heparin and protamine. For example, Guo designed a voltammetric sensor for the detection of heparin at polarized blood plasma/1,2-Dichloroethane interfaces [9]. Fu and co-workers reported a colorimetric sensor for the detection of heparin based on self-assembly of gold nanoparticles [10] and the color quenching of gold nanorods [11]. Egawa designed a fluorometric method for the determination of heparin based on self-quenching of fluorescein-labeled protamine [12]. Shvarev described a reversible electrochemical sensor for protamine [13]. Awotwe-Otoo reported a robust reverse phase-HPLC method to quantify protamine [14]. Wang and co-workers developed a lipophilic dichlorofluorescein derivatives optical films for protamine detection [15]. However, these methods are generally limited to the detection limit and require relatively expensive and complicated instruments. Furthermore, only one analyte could be tested with these methods. At present, there are very few methods established for the determination of heparin and protamine by one sensor. For example, Gemene et al. developed a chronopotentiometric sensor for detection of the polyions protamine and heparin [16]. This method was rapid and sensitive, but it required complicate electrode modification process. Colorimetric sensor with nano-sized gold particles has been used for the determination of

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heparin and protamine [1]. It is simple and easily read out by naked-eyes. However, the sensitivity is not enough to meet the assay requirements.

Here, we take the advantage of high sensitivity of fluorescence-based optical biosensor [17–19], and the high extinction coefficience and excellent fluorescence quenching ability of gold nanoparticles (AuNPs) [20–22] to develop a novel method for sequential detection of heparin and protamine. The emission spectrum of fluorescein (the emission maximum at 520 nm) overlaps the absorbance band of AuNPs (the absorbance maximum at 520 nm). Thus highly efficient fluorescence resonance energy transfer (FRET) would occur between them [23]. Protamine can compete with fluorescein molecules and be adsorbed onto the surface of the AuNPs, resulting in the detaching of the adsorbed fluorescein molecules from the surface of AuNPs and the restore fluorescence of the fluorescein. In the existence of heparin, protamine has the priority to interact with heparin over AuNPs, which resulted in the de-aggregation of AuNPs and the recombination of AuNPs with fluorescein molecules, than the quench of fluorescein fluorescence reoccurs. The proposed detection strategy is shown in Fig. 1. The experimental results demonstrate that this assay is a simple, sensitive method to detect heparin and protamine sequentially.

2. Experimental

2.1. Materials

Chloroauric acid ($HAuCl_4$), fluorescein, protamine sulfate salt and heparin sodium salt were purchased from Sigma (Shanghai, China). All other chemicals (99%, Merck) used in this work were of analytical grade and Millipore Milli-Q ultrapure water (Millipore, ≥ 18 MΩ cm) was used throughout the experiments.

2.2. Instruments

The absorption spectra of AuNPs were recorded on a UV-2450 spectrophotometer (Shimadzu Co., Japan). An F-4500 fluorescence spectrophotometer (Hitachi Co., Japan) was used to collect the fluorescent emission spectra of the fluorescein. Transmission electron microscopy (TEM) images were collected on a JEOL-1230 transmission electron microscope (JEM-3010 Joel, Japan). The HPLC system comprised a binary high-pressure pump LC-20AT, a SPD-20AV UV–vis spectro-photometric detector (Shimadzu, Japan).

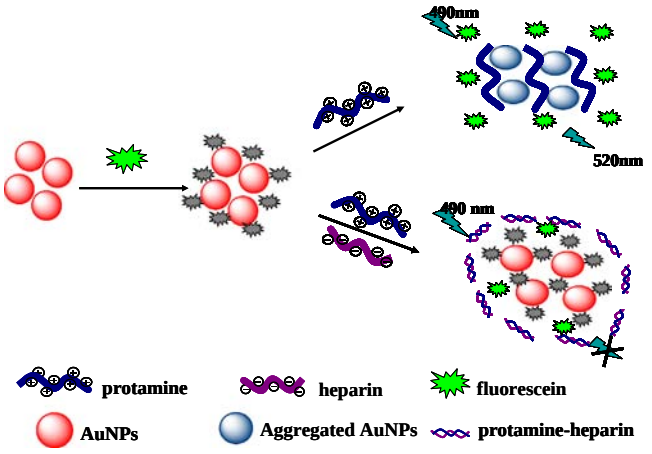

Fig. 1. Scheme of the detection of protamine and heparin based on the dissolution of fluorescein–AuNPs. The fluorescence of fluorescein is recovered and protamine induced aggregation of AuNPs occurs when protamine is incubated with fluorescein–AuNPs mixture. The fluorescence of fluorescein is re-quenched and heparin driven de-aggregation of AuNPs take place when heparin and protamine is incubated with fluorescein–AuNPs mixture.

2.3. Synthesis of AuNPs

AuNPs were prepared according to the literature [24]. Briefly, 50 mL chloroauric acid ($HAuCl_4$) solution (containing 1.67 mL 1% $HAuCl_4$) was firstly heated to boiling, and then 5 mL of 38.8 mmol/L sodium citrate solution was rapidly added to the boiled $HAuCl_4$ solution under vigorous stirring. The mixed solution was boiled for 10 min and further stirred without heating for another 15 min. The obtained wine-red solution was cooled to room temperature and stored in the refrigerator (4 °C) for further use. The concentration of the AuNPs was estimated by Beer's law (the molar extinction coefficient for 13 nm AuNPs is 2.78×10^{-8} mol/L). The size of AuNPs was measured by TEM.

2.4. The detection procedure of protamine and heparin

100 μL of AuNPs and 100 μL of fluorescein solution (the final concentration is 3.2×10^{-7} mol/L) were incubated in the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (800 μL 10 mmol/L) to form fluorescein–AuNPs complex. Then different concentrations of protamine (50 μL) were incubated with fluorescein–AuNPs mixture (the final volume is 1 mL). Heparin was detected with the following steps: protamine (0.8 μg/mL) was added to fluorescein–AuNPs mixture and then different concentrations heparin was added to the resulted solution (the total volume is 1 mL). After incubation for 90 min, fluorescence emission spectra were recorded with excitation wavelength at 480 nm.

3. Results and discussion

3.1. Fuorescein fluorescence quenching by AuNPs

Fig. 2 shows the absorption spectrum of AuNPs and the excitation and emission spectra of fluorescein. It was found that the surface plasmon resonance (SPR) and absorbance band of the AuNPs (curve a) is located at ranges from 515 to 530 nm (curve c), and the emission maximum of fluorescein fluorescence locates at approximately 520 nm (curve b). The emission spectrum of fluorescein was overlapped with the surface plasmon resonance (SPR) scattering and absorbance band of the AuNPs. Therefore, the FRET and inner filter effect (IFE) could be occurred between AuNPs and fluorescein and the fluorescence of fluorescein could be quenched. As expected, upon addition of AuNPs to the fluorescein solution,

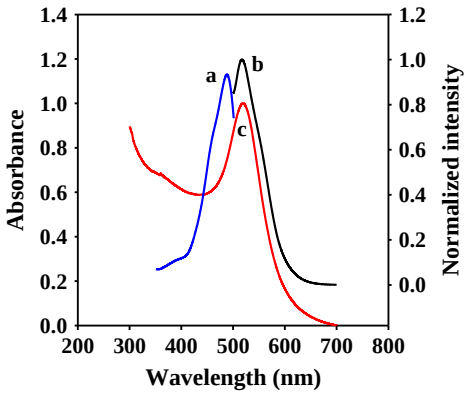

Fig. 2. Excitation spectrum (a) and emission spectrum (b) of fluorescein and absorption spectrum of AuNPs (c).

the fluorescence of fluorescein was promptly quenched (as shown in Fig. S1 in the supporting information), which indicated the fluorescein molecule adsorbed onto the surface of citrate-stabilized AuNPs to form a fluorescein–AuNPs assembly due to the electrostatic interaction. We estimated the effect of FRET and IFE on fluorescence quenching according to the literature [23] (see the detailed discussion in supporting information). The relative contribution of IFE and FRET to the fluorescence quenching could be 15% and 85% by rough estimation, respectively. The result suggested that both FRET and IFE contributed to the observed fluorescence decrease of fluorescein in the presence of AuNPs, while the FRET was the dominant factor. The fluorescent quenching data shown in Fig. S1 was analyzed according to the Stern–Volmer equation [23],

$$I_0/I = K_{sv} \times [Q] + 1 \quad (1)$$

where I_0 and I are the fluorescent intensity of fluorescein in the absence and presence of AuNPs, respectively. [Q] is the concentration of the quencher, AuNPs. The relationship between I_0/I and the [AuNPs] was shown in Fig. S2 (in the supporting information). The data were analyzed using the Stern–Volmer quenching plot:

$$I_0/I = 0.3 \times [Q] + 1 (R^2 = 0.99) \quad (2)$$

where the unit of [Q] is nmol/L, and the quenching constant (K_{sv}) was estimated to be $3 \times 10^{10}\ M^{-1}$. This large quenching constant suggests that AuNPs efficiently quench the fluorescence of fluorescein.

3.2. Investigation of interaction between analytes and fluorescein–AuNPs assembly

It is well know that protein, such as protamine, can react with AuNPs easily. Fluorescein molecules adsorbed on the surface of AuNPs could be released after introducing protein in the detection system, which will result in the recovery of the fluorescence of fluorescein. To validate the competition effect between fluorescein and protamine, we detected the fluorescence of the system before and after adding protamine, the results were shown in Fig. 3E. When fluorescein was mixed with AuNPs, the fluorescence of fluorescein was quenched by AuNPs (curve b). The fluorescence was re-enhanced after adding protamine to fluorescein–AuNPs mixture (curve c). And the fluorescence was quenched again after heparin was added to the test system (curve d), owing to the prior interaction of protamine with heparin over AuNPs, which resulted in the de-aggregation of AuNPs. The TEM images of AuNPs and the color changes of the test solution (Fig. 3) recorded at different stage of the above test procedure, which proved the interaction mechanism of fluorescein, protamine and heparin with AuNPs. As shown in Fig. 3A, the free AuNPs with about 13 nm in diameter was in a dispersed state, which renders the red color to the colloidal AuNPs. In the presence of fluorescein, AuNPs was retained in a dispersed state and the solution kept the red color (Fig. 3B). While protamine was added into the solution of fluorescein-AuNPs assembly, a rapid red-to-blue color change of the solution took place and AuNPs were obviously aggregated (Fig. 3C). When heparin was further added into the test system, heparin-driven de-aggregation of aggregated AuNPs occurred and the color of the solution turned to shallow purple (Fig. 3D). These changes in the morphology of AuNPs and color of the solution during the test procedure revealed the strong interaction of fluorescein, protamine and with AuNPs and the strong interaction of protamine with heparin. These observations also revealed the order of the interactions. The descending order of the priority should be heparin with protamine, protamine with AuNPs, fluorescein with AuNPs. The observations are consistent with that obtained from fluorescence measurement.

3.3. Optimization of the detection conditions

In order to obtain a high sensitive sensor for the detection of protamine and heparin, the optimization of the conditions is essential. The detection conditions were optimized in two stages: the detection of protamine and sequential detection of heparin. Firstly, we investigated the effect of the concentration of AuNPs. As shown in Fig. 4A, the fluorescent enhancement efficiency in the presence of protamine was gradually increased with the concentration of the AuNPs. The maximum was obtained at 3 nmol/L AuNPs, and the fluorescent enhancement efficiency decreased

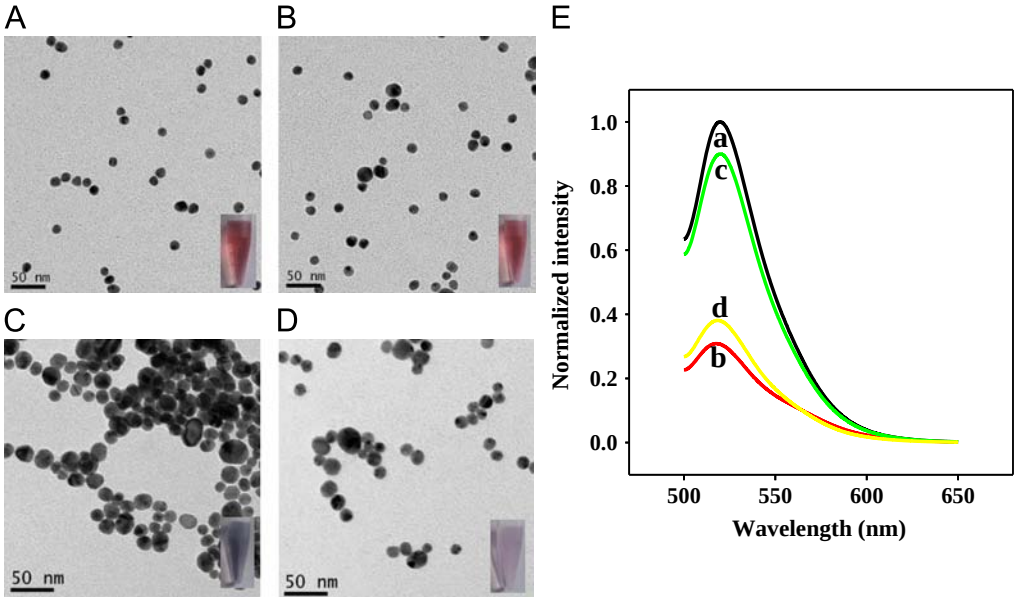

Fig. 3. TEM images of AuNPs: free AuNPs (A), in the presence of fluorescein (B), in the presence of fluorescein and protamine (C) and in the presence of fluorescein, protamine and heparin (D). Fluorescence spectra (E): fluorescein(a). fluorescein and AuNPs(b). mixture of AuNPs, fluorescein and protamine(c). fluorescein-AuNPs incubated with heparin and protamine(d). [fluorescein]: 3.2×10^{-7} mol/L, [AuNPs]: 3 nmol/L, [protamine]: 0.8 μg/mL and [heparin]: 1.4 μg/mL.

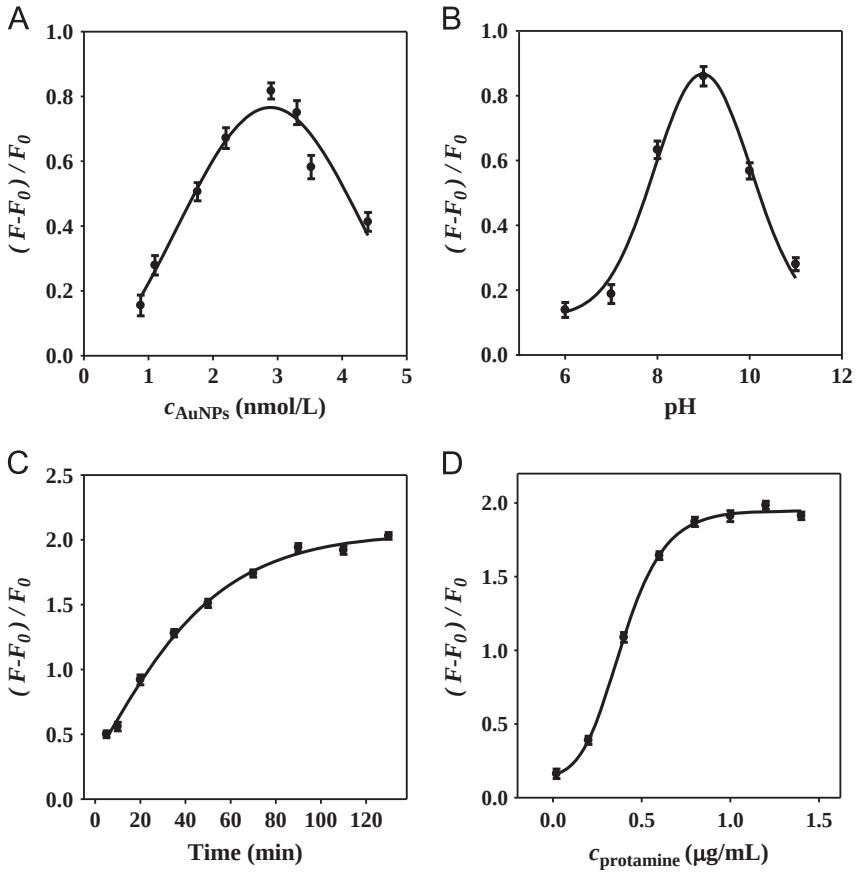

Fig. 4. Effects of AuNPs concentration (A), pH (B) and incubation time (C) on the fluorescence responses of FRET sensor for protamine detection. Effects of protamine concentration on the fluorescent sensor for heparin detection (D).

when the concentration of the AuNPs was beyond 3 nmol/L. Thus, 3 nmol/L of AuNPs was chosen for the following experiment.

The fluorescence intensity of fluorescein is highly dependent on solution pH. The effect of pH on the efficiency of fluorescence enhancement was investigated in the range from pH 6.0 to 11.0. As shown in Fig. 4B, the highest enhancement efficiency of fluorescence was obtained at pH 9.0. Therefore, the ideal pH value for protamine detection is 9.0. The effect of the incubation time of protamine was investigated as well. Fig. 4C shows the time-dependent fluorescent response in the presence of protamine. The fluorescence response remained a stable value when the incubation time reached to 90 min, suggesting that the competitive reaction of protamine and fluorescein reached equilibrium within 90 min. Therefore, 90 min was employed as the incubation time of protamine. For optimization of the detection conditions of heparin, we further studied the effect of the amount of protamine. Fig. 4D shows the effect of the concentration of protamine on the fluorescence response. It is found that the fluorescence response increase rapidly with the concentration of protamine and reached a stable value when the concentration of protamine was 0.8 μg/mL. Therefore, 0.8 μg/mL was chosen as the optimal concentration of protamine for heparin detection.

3.4. The detection of protamine and heparin

Under the optimized conditions, the capability of this analytical system for quantitative detection of protamine and heparin was evaluated. The fluorescent spectra of fluorescein in the presence of AuNPs and different amounts of protamine were shown in Fig. 5. The fluorescence intensity was increased with the concentration of protamine and linear range for the concentration of protamine was 0–0.8 μg/mL with a detection limit of 0.0067 μg/mL ($S/N=3$). The linear regression equation was $F=1.2926C+0.0376$ with the correlation coefficient (R^2) of 0.99, where F refers to the measured fluorescence intensity and C refers to the concentration of protamine. Fig. 6 shows the fluorescence spectra of fluorescein–AuNPs mixture solution containing 0.8 μg/mL protamine and different concentration of heparin. A linear relationship between the fluorescence intensity and the concentration of heparin in the range of 0.004–1.6 μg/mL was obtained. The linear regression equation was $F=-0.3498C+0.9590$ with the correlation coefficient (R^2) of 0.99, where F refers to the measured fluorescence intensity and C refers to the concentration of heparin. The limit of detection (LOD) was calculated to be 0.0013 μg/mL ($S/N=3$). These detection limit values are lower than that of the reported fluorometry for protamine and heparin and the linear response ranges at low concentration are wider than reported (Table S1).

3.5. Selectivity

In order to evaluate the selectivity of the sensor for the detection of protamine and heparin, the interference from common cations and proteins was investigated. Comparative trials were carried out using Na^+, Ca^{2+}, Mg^{2+}, K^+, lysozyme and IgG as the potential interferents. Fig. 7A shows the changes in the fluorescence intensity of the sensor in the presence of other species. The results show that these species led to only minor response. Fig. 7B shows the fluorescence spectra of the fluorescein–AuNPs/protamine mixed solution after the addition of analogs of heparin such as hyaluronic acid (HA), dextran (Dex) and

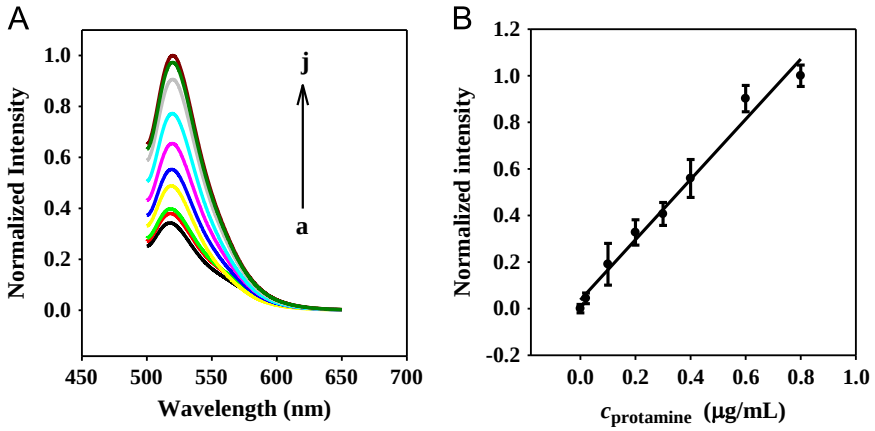

Fig. 5. (A) Fluorescent spectra of fluorescein–AuNPs mixture in the presence of different concentration of protamine. The concentration of protamine (from bottom to top) is 0, 2×10^{-8}, 4×10^{-8}, 1×10^{-7}, 2×10^{-7}, 3×10^{-7}, 4×10^{-7}, 6×10^{-7}, 8×10^{-7} and 1.2×10^{-6} g/mL respectively. (B) The linear fitting of the fluorescence response vs. protamine concentration.

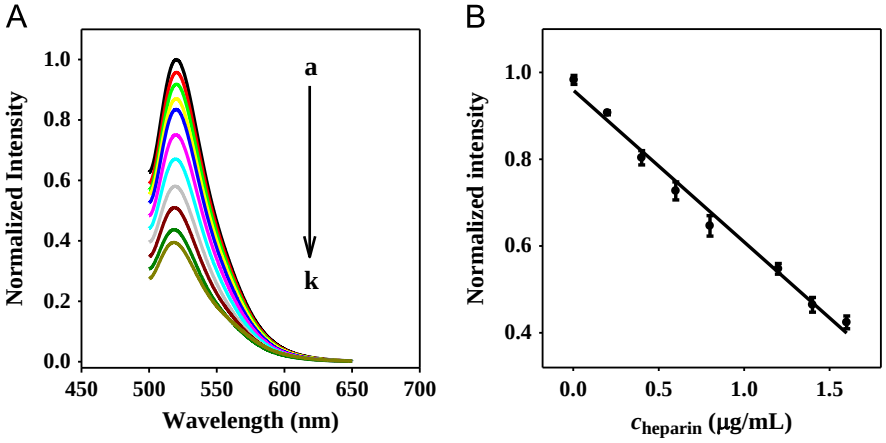

Fig. 6. (A) Fluorescent spectra of fluorescein–AuNPs /protamine mixed solution with various concentration of heparin. The concentration of heparin (from bottom to top) is 0, 4×10^{-9}, 2×10^{-8}, 1×10^{-7}, 2×10^{-7}, 4×10^{-7}, 6×10^{-7}, 8×10^{-7}, 1.2×10^{-6}, 1.4×10^{-6}, 1.6×10^{-6} g/mL respectively. [protamine]: 0.8 μg/mL. (B) The linear fitting of the fluorescence response vs. concentrations of heparin.

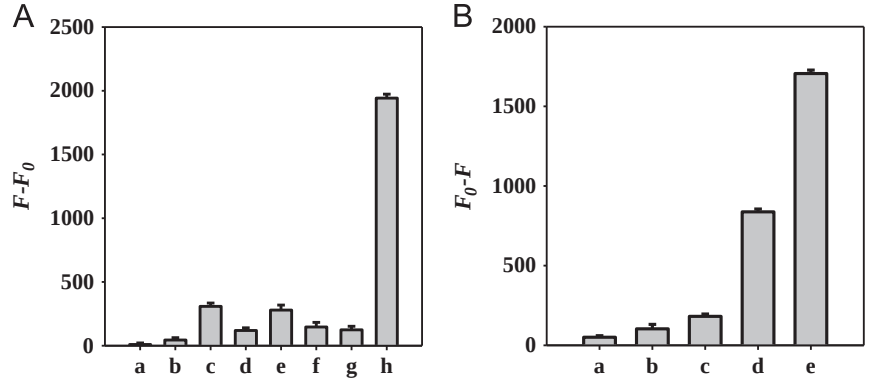

Fig. 7. (A) Fluorescent enhancement efficiency of fluorescein-AuNPs mixture upon the addition of protamine or other substances: (a) blank; (b) Mg^{2+} (0.16 mg/mL); (c) IgG (0.2 mg/mL); (d) Na^+ (2 mg/mL); (e) Ca^{2+} (0.11 mg/mL); (f) lysozyme (0.2 mg/mL); (g) K^+ (2 mg/mL); (h) protamine (0.06 mg/mL). (B). Fluorescent enhancement efficiency of fluorescein–AuNPs/protamine mixed solution upon addition of heparin or other substances: (a) blank; (b) Dex (1.4 μg/mL); (c) HA (1.4 μg/mL); (d) Chs (1.4 μg/mL); (e) heparin (1.4 μg/mL).

chondroitin sulfate (Chs). The concentration of protamine was 0.8 μg/mL. Obviously, the fluorescence intensity has a slight variation compared to the blank in the presence of HA and Dex. Heparin led to a remarkable fluorescence intensity decrease at an identical concentration with HA, Dex and Chs. As shown in Fig. 7B, Chs maybe have a influence on detection of heparin. However, Chs mainly exists in animal cartilage tissue [25]. Normally, the detection of heparin was conducted in serum. Therefore, the interference of Chs can be neglected for the detection of

Table 1
Detection of protamine in 10% serum. (a) Recovery refers to the ratio of |Calculated/Added|. Calculated means the values that we get according to the linear standard curve equations. Added means the values that we add into 10% serum.

Sample	Added (μg/mL)	Calculated (μg/mL)	Recovery (%)	RSD (%)
1	0.20	0.21	105.00	0.31
2	0.40	0.39	97.50	0.21
3	0.60	0.58	96.67	0.44

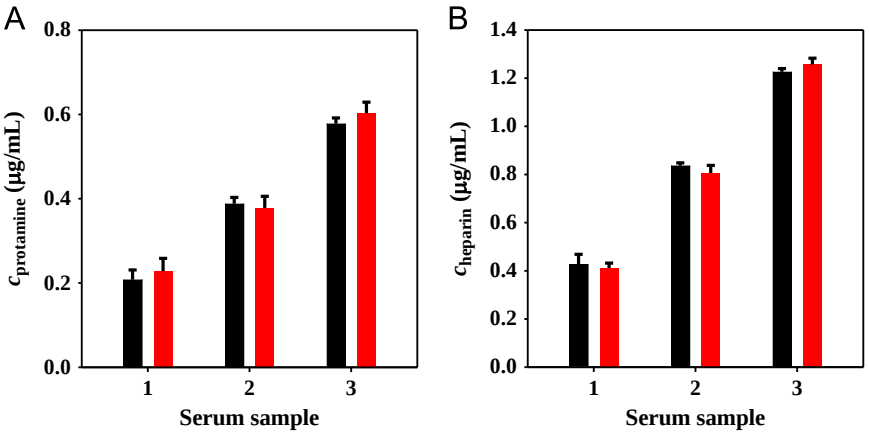

Fig. 8. Analytical results of protamine and heparin in serum obtained using the present method (black) and by HPLC (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Detection of heparin in 10% serum. (a) Recovery refers to the ratio of |Calculated/Added|. Calculated means the values that we get according to the linear standard curve equations. Added means the values that we add into 10% serum.

Sample	Added (μg/mL)	Calculated (μg/mL)	Recovery (%)	RSD (%)
1	0.40	0.43	107.50	1.77
2	0.80	0.84	105.00	0.96
3	1.20	1.23	102.50	0.72

heparin in serum. These results demonstrated that the developed method provided attractive specificity for heparin detection.

3.6. Application of the sensor

To demonstrate the practical utility of the developed sensor, it was further applied to detect protamine and heparin in a real blood serum sample. The serum sample was diluted 10-fold and then a known quantity of heparin or protamine was added to the sample. The fluorescence of the test system was detected following by the proposed strategy to record the fluorescence response. The results are shown in Table 1. The experimental results exhibited that recoveries of protamine and heparin reached to 96.67–105% and 102.5–107.5%, with the relative standard deviation (RSD) of 0.21–0.44% and 0.72%–1.77% respectively. As the recoveries of the samples were very satisfied, it demonstrated that the proposed method can be applied to detection protamine and heparin in real samples. In order to further demonstrate the accuracy of this method, the concentration of protamine and heparin in each sample was determined by the proposed assay and analyzed by the HPLC according to the literature [14,26]. The concentrations for protamine and heparin in serum which were detected by the proposed assay and HPLC were shown in Fig. 8. The results obtained by this assay are consistent with those obtained from HPLC, indicating that the proposed method can be used to analyze heparin and protamine accurately Table 2.

4. Conclusions

In summary, a sensitive fluorescent sensor for the detection of heparin and protamine was developed. The AuNPs was used as a quencher for the fluorescein fluorescence. Due to the excellent fluorescence quenching ability of AuNPs, excellent detection sensitivity for protamine and heparin was obtained with this novel fluorometry for protamine and heparin. Moreover, this fluorescenct sensor can be used to distinguish heparin and protamine from their analogs. This selective sensing system offered the superiorities of label-free, simplicity and high sensitivity for the detection of heparin and protamine. We believe that the fluorescent sensor will provide a versatile tool for the determination of heparin and protamine in wide areas.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.08.010.

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