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A simple and rapid label-free fluorimetric biosensor for protamine detection based on glutathione-capped CdTe quantum dots aggregation

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ABSTRACT

A novel fluorescent biosensor is developed, based on glutathione-capped CdTe quantum dots aggregation, for the determination of trace amount of an important drug, protamine. In this method with increasing the protamine concentration, the fluorescence of the quantum dots was quenched due to their aggregation. Different parameters affect the sensitivity, such as pH and the amount of the quantum dots, were optimized. Using the new optical biosensor, under the optimized conditions, protamine could be measured in the range of 2.0–200 ng mL^{-1} with a detection limit of 1.0 ng mL^{-1}. The relative standard deviation for five replicates determination of 30.0 ng mL^{-1} protamine was 1.26%. The influence of common interfering species on the protamine detection was studied. The results showed that the biosensor is highly selective and sensitive for the detection of protamine. The optical biosensor was successfully used for the determination of protamine in real samples.

1. Introduction

Protamine is a low molecular weight protein (Vasimalai and Abraham John, 2013). It is a highly cationic peptide and has 20-positive charge in physiological condition (Vasimalai and Abraham John, 2013). Protamine is separated from the sperm of salmon and certain other species of fish. For the first time, protamine is produced mostly by recombinant biotechnology (Carr and Silverman, 1999). Protamine is an important pharmaceutical compound that is used in cardiac surgery, vascular surgery and interventional radiology procedures as an antidote for heparin (a drug used to treat and prevent blood clots) overdose. Common unfavorable effects of protamine include sudden fall in blood pressure, bradycardia, pulmonary hypertension, dyspnea, or flushing and a feeling of warmth (Carr and Silverman, 1999). For these reasons, rapid and accurate analytical methods for protamine determination in plasma samples have attracted much attention.

Several methods have been reported for the protamine measurement, including high performance liquid chromatography (Snycerski et al., 1998; Hvass and Skelbaek-Pedersen, 2005), microtiter plate-format optodes (Kim et al., 2001; Wang et al., 1996; Dai et al., 2000), electrochemical sensors (Gadzekpo et al., 1999; Xiao et al., 2001) and fluorescence assay (Pang et al., 2014). Recently fluorometric (Vasimalai and Abraham John, 2013; Zhao et al., 2013) and colorimetric (Jena and Raj, 2008) methods have been revealed for the quantification of protamine based on aggregation of gold nanoparticles (AuNPs). A few fluorescence assays have been developed based on aggregation/disaggregation of quantum dots (QDs) for the determination of biomolecules, such as neurogenin3 (Yuan et al., 2012) and fluoride (Liu et al., 2011), detection of DNA sequences (Kim et al., 2009) and proteomic antigens (Soman and Giorgio, 2009).

Research on semiconductor nanocrystals, also known as QDs, has increased fast in the past few decades (Petryayeva et al., 2013). The main attractions for the use of fluorescent QDs in bioanalysis and bioimaging are their optical and chemical properties such as superior photostability, excellent resistance to chemical and photochemical degradation, wide absorption spectra, narrow photoluminescence spectrum and good fluorescence quantum yield (Bharali et al., 2005).

Herein, a novel biosensing method for protamine detection was developed based on glutathione-capped cadmium telluride quantum dots (CdTe-QDs), as a fluorescent probe, by the aggregation-based fluorescence property. As it will be demonstrated in this study, the new optical biosensor has many advantages such as high sensitivity with fast response in the detection of protamine. This sensor can detect very low concentrations of protamine without any pre-concentration steps.

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2. Experimental

2.1. Reagents and chemicals

All chemicals and reagents with a highest degree of purity available were obtained and doubly distilled water was used throughout. Glutathione, sodium tellurite, cadmium chloride, protamine sulfate and sodium borohydride were purchased from Aldrich (London, UK).

A stock solution of 50.0 μg mL^{-1} protamine was prepared by dissolving an appropriate amount of protamine sulfate in water into a 100 mL standard flask. Lower protamine concentrations were prepared by sequential dilution of the stock solution. Universal buffer solution, including boric acid/citric acid/phosphoric acid (0.04 mol L^{-1} each) was used. The final pH of the buffer solutions were adjusted by the addition of 0.2 mol L^{-1} sodium hydroxide.

2.2. Apparatus and measurements

UV absorption spectra were recorded on a Jasco V-570 UV/vis/NIR spectrophotometer (Tokyo, Japan). Photoluminescence spectra were recorded on a Jasco FP-750 spectrofluorometer (Tokyo, Japan). Transmission electron microscopic (TEM) measurements were conducted on a Philips (The Netherlands), Model CM30 TEM, operating at 300 kV. X-ray diffraction (XRD) pattern of vacuum-dried GSH-CdTe powder of GSH-CdTe QDs was performed on a Philips XL-30 ESEM (The Netherlands). The particle size distribution of GSH-CdTe QDs in aqueous solution was acquired using a Malvern ZEN3600 dynamic light scattering instrument (UK).

2.3. Preparation of water-soluble glutathione-capped CdTe-QDs

Synthesis of water-soluble glutathione-capped CdTe-QDs was performed according to the Cui et al. (2012) method. In a typical synthesis, first, 2.0 mL of 0.040 mol L^{-1} $CdCl_2$ solution was diluted to 50 mL with water. Then, 0.050 g of trisodium citrate dihydrate, 0.025 g of glutathione and 0.025 g of $NaBH_4$ plus 2.0 mL of Na_2TeO_3 (0.01 mol L^{-1}) were added to the above solution under vigorous stirring at room temperature, during 2 h. Then, the mixture was refluxed for 12 h at 90 °C. The resulting solution was transferred to a dark container and then was kept at 4 °C. According to the absorption spectrum the amount of glutathione-capped CdTe-QDs was calculated as 0.34 μmol L^{-1} (Yu et al., 2003).

2.4. Fluorescent assays

For the determination of protamine, a freshly prepared mixture containing 2.8 nmol L^{-1} of glutathione-capped CdTe-QDs and an appropriate volume of the sample solution were mixed and buffered with the universal buffer (pH 11.0). Then, the fluorescence intensity of the solution was recorded at an excitation wavelength of 400 nm and the emission spectrum was recorded in the range of 550–700 nm. The fluorescence spectrum was recorded using an excitation slit of 10.0 nm and emission slit of 10.0 nm. The response function ($F_0 - F$) values of the biosensor were obtained with different concentration of protamine, where F_0 and F are the fluorescence intensities at 620 nm in the absence and presence of protamine, respectively.

2.5. Sample preparation

Human blood plasma were filtered to remove any particles and 10 times were diluted before analysis. Standard addition method was used for the determination of protamine. An appropriate amount of standard protamine solution was spiked into the human serum. After its filtration, 300 μL of the spiked serum sample was added to 3 mL of the sensing solution (containing 2.8 nmol L^{-1} of glutathione-capped CdTe-QDs, pH 11.0). Then, the fluorescence spectrum was recorded at 550–700 nm upon excitation at 400 nm.

3. Results and discussion

3.1. Principle of the operation

Modifying the outer surface of QDs with anionic carboxylate groups is one of the most commonly used methods for dispersing QDs in aqueous solution (Petryayeva et al., 2013). For this purpose, glutathione was used. At adequately basic pH, electrostatic repulsion between QDs affords a stable colloidal suspension. TEM image of glutathione-capped CdTe-QDs (Fig. 1A) shows much less agglomerated particles before protamine addition, with average particle size of 7–8 nm.

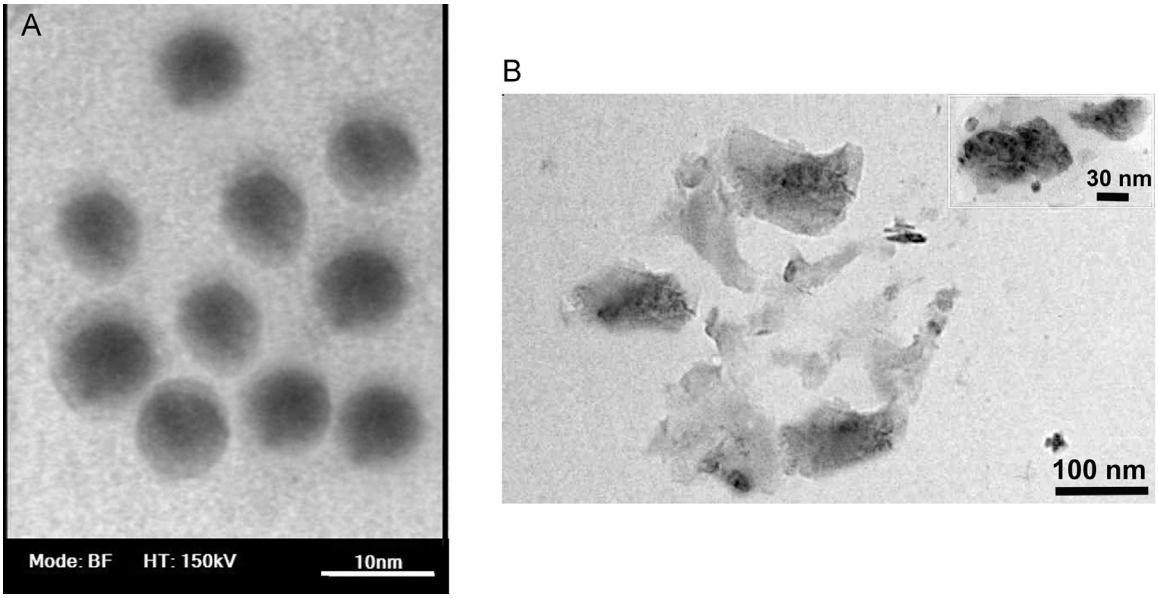

Fig. 1. TEM images of glutathione-capped CdTe-QDs (A) before addition of protamine; and (B) after protamine addition.

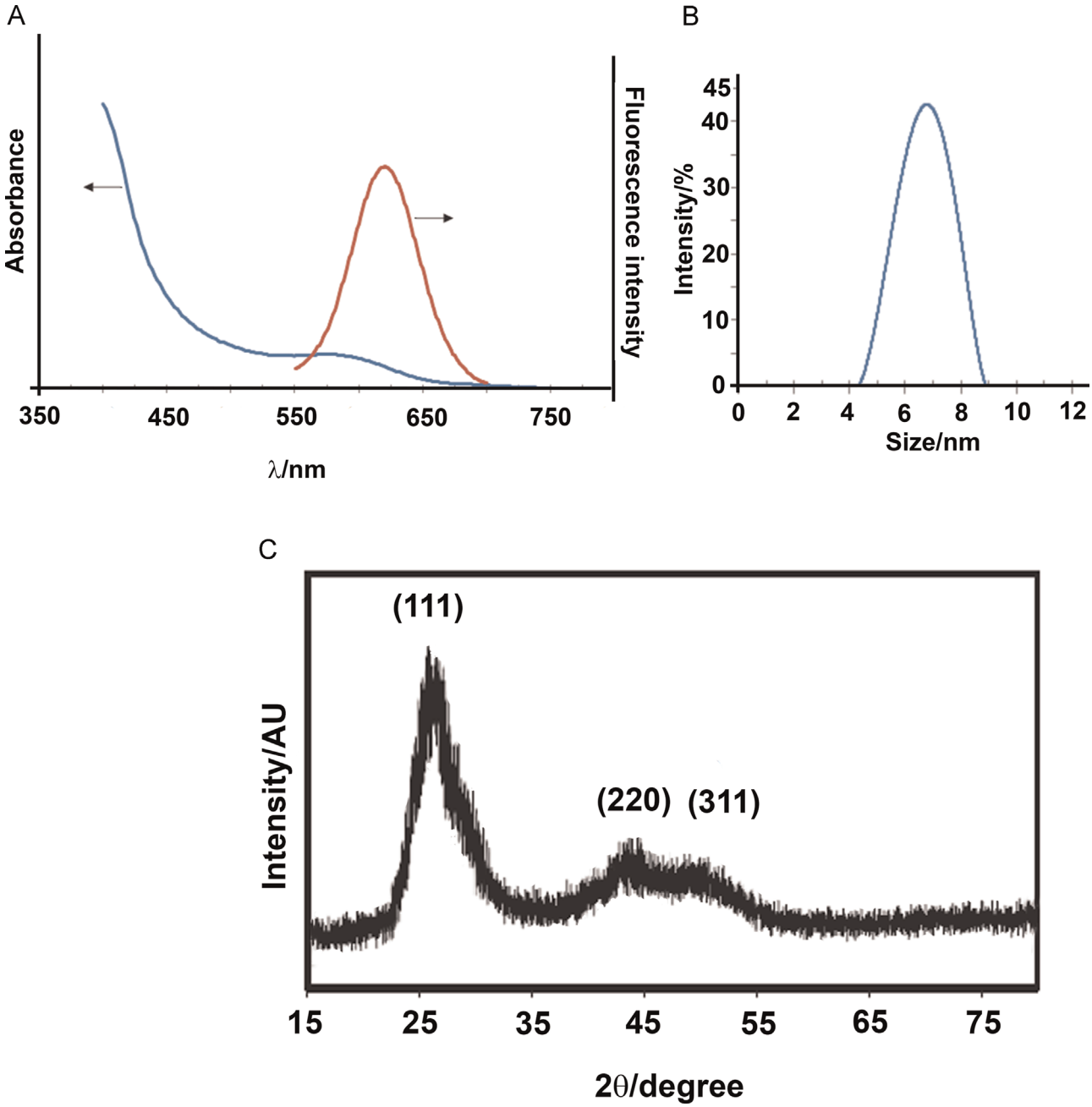

Fig. 2. (A) Absorbency and fluorescence spectrum of CdTe-QDs. (B) DLS analysis of CdTe-QDs. (C) XRD pattern of the CdTe-QDs.

The surface charge of glutathione-capped CdTe-QDs was negative at pH 11, whereas protamine has positive charge (Zhang et al., 2012; Zhao et al., 2013). Fig. 1B shows the TEM image of glutathione-capped CdTe-QDs after protamine addition. As can be seen, the QDs aggregation takes place when protamine is added. The electrostatic interaction between negatively charged QDs and positively charged protamine could reduce the electrostatic repulsion forces between the QDs by neutralizing the surface charges. This explains the QDs aggregation by the addition of protamine. Upon aggregation of the QDs, the fluorescence was quenched and a red-shift in the fluorescence spectrum (~10 nm) could be seen. This is due to the fact that the average inter-QD distance becomes much shorter. Significantly, the energy transfer from the smaller QDs (higher energy emission) to the larger QDs in the ensemble (lower energy emission) can take place (Wuister et al., 2003).

Fig. 2A shows the absorbance and emission (fluorescence) spectra of the QDs. As shown in the spectrum, the fluorescence spectrum is appeared between 550 and 700 nm. Dynamic light scattering (DLS) was used to check the size distribution of the GSH-CdTe QDs in aqueous solution. As shown in Fig. 2B, the QDs nanoparticles in aqueous solution have a narrow size distribution of 4.5–9.0 nm. X-ray diffraction (XRD) was also used to check the crystal structure of the GSH-CdTe QDs. The X-ray pattern of GSH-CdTe powder (Fig. 2C) showed a band at ca. 45° due to overlap of (311) and (220) diffraction and a peak at 26° (111). These peaks confirm the crystal structure of GSH-CdTe QDs (Zheng et al., 2007).

3.2. Effect of sample solution pH

The relationship between the fluorescence intensity and the solution pH was investigated in the presence of 100.0 ng mL^{-1} protamine. For this purpose, the response function ($F-F_0$) values

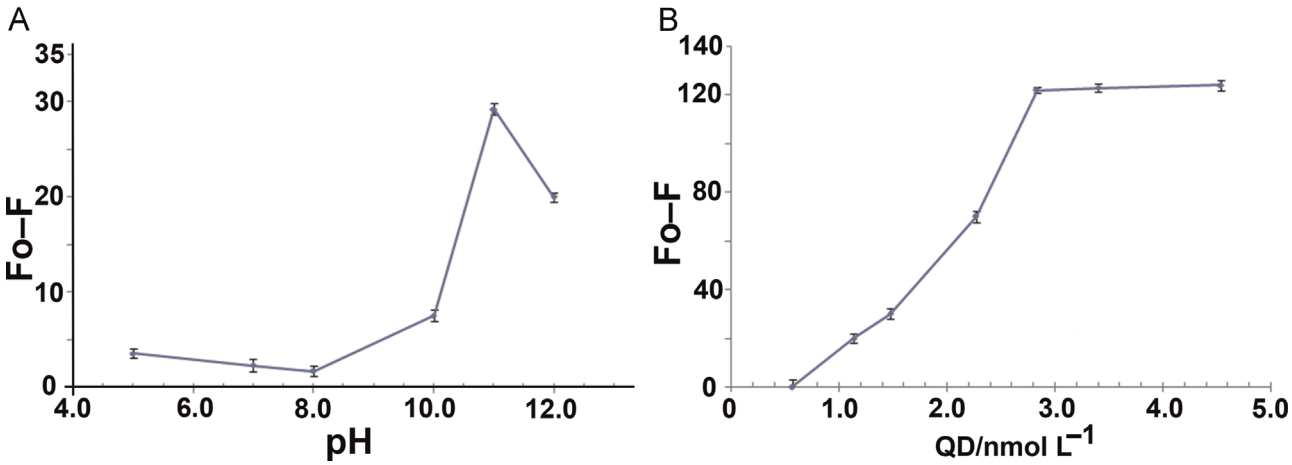

Fig. 3. (A) Effect of pH on the sensor response. Conditions: 1.5 nmol L^{-1} of glutathione-capped CdTe-QDs and 100.0 ng mL^{-1} of protamine at different pH values. (B) Influence of the amount of glutathione-capped CdTe-QDs on the response of the biosensor to protamine ions. Conditions: 100.0 ng mL^{-1} of protamine at pH 11.0 with different amount of glutathione-capped CdTe-QDs. The error bars show the relative standard deviations ($n=3$).

of the biosensor were obtained. Different pH values (5.0–12.0) were tested using solutions containing 1.5 nmol L^{-1} of glutathione-capped CdTe-QDs and 100.0 ng mL^{-1} protamine (Fig. 3A). As can be seen, pH 11.0 is the best pH value for determination of protamine by the proposed method. This is due to the fact that pK_a of the –COOH group in glutathione is 3.6 thus, the zeta potential of the glutathione-capped CdTe-QDs was negative at pH 11.0. At adequately basic pH, the electrostatic repulsion between QDs affords a stable colloidal suspension, while in acidic pH the photoluminescence emission intensity of glutathione-capped CdTe-QDs was totally quenched (Petryayeva et al., 2013). Protamine is extremely positive protein with an isoelectric point of pH 12–13 (Zhao et al., 2013), and it has positive charge at pH 11.0. Thus, the interaction between protamine and glutathione-capped CdTe-QDs at pH 11.0 was electrostatic type. Nevertheless, at pH >11, the response intensity of the biosensor was decreased. This is due to the fact that at pH >11 protamine has negative charge so could not aggregate the glutathione-capped CdTe-QDs. Therefore, an optimum pH of 11.0 was selected for further experiments.

3.3. Effect of glutathione-capped CdTe-QDs

It was found that the concentration of glutathione-capped CdTe-QDs affect the the method sensitivity. As shown in Fig. 3B, where the concentration of glutathione-capped CdTe-QDs increased in the presence of 100.0 ng mL^{-1} protamine at pH 11.0, the sensitivity increased considerably. In addition, if the concentration was too low, the fluorescence intensity was also very low, which may sacrifice the linear calibration range. Based on the obtained results, 2.8 nmol L^{-1} of glutathione-capped CdTe-QDs was selected as an optimum concentration of the QDS for further study.

4. Figures of merit

Under the optimal conditions described above, the fluorimetric spectra of glutathione-capped CdTe-QDs with different concentrations of protamine were recorded (Fig. 4A). The response function (F_0-F) values of the biosensor were obtained with different protamine concentrations. Fig. 4B shows the calibration curve of protamine at the optimum conditions. A linear range of 2.0–200 ng mL^{-1} protamine was obtained with a correlation coefficient of 0.993.

The theoretical limit of detection (LOD) was obtained as 0.2 ng mL^{-1} (LOD$=3S_b/m$, where S_b is the standard deviation of the blank measurements ($n=10$) and m is the slope of the calibration graph), whereas the experimental limit of detection was

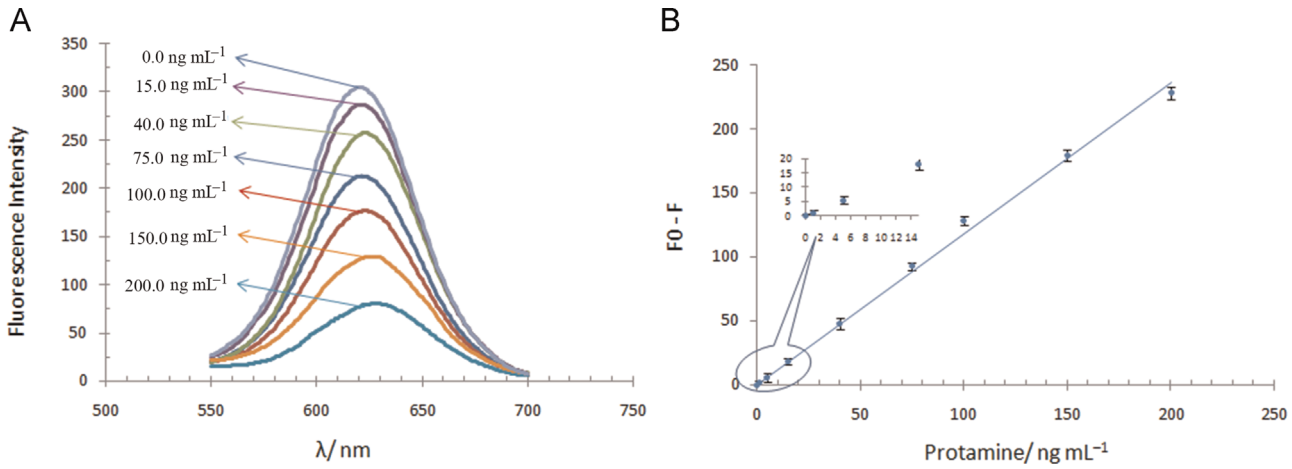

Fig. 4. (A) Fluorescence spectra of the biosensor in the presence of different amounts of protamine concentration. Conditions: 2.8 nmol L^{-1} of glutathione-capped CdTe-QDs at pH 11.0 containing different concentration of protamine as: 1) 0.0; 2) 15.0; 3) 40.0; 4) 75.0; 5) 100.0; 6) 150.0 and 7) 200.0 ng mL^{-1}. (B) Calibration graph for the protamine determination at the optimum conditions. The error bars show the relative standard deviations ($n=3$).

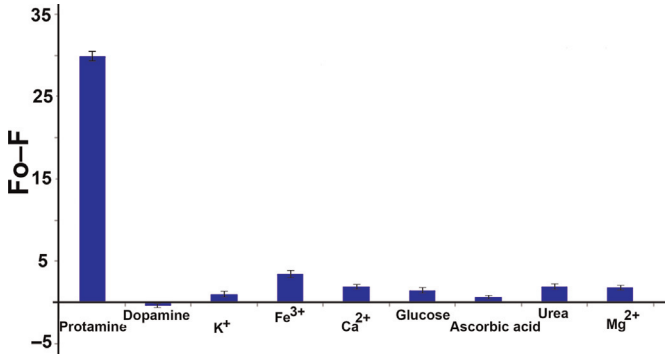

Fig. 5. Fluorescence responses of a mixture containing 2.8 nmol L^{-1} of glutathione-capped CdTe-QDs at pH 11.0 in the presence of different species at 15000 ng mL^{-1} and 30.0 ng mL^{-1} protamine. The error bars show the relative standard deviations ($n=3$).

Table 1
Determination of protamine in real samples.

Human serum samples	Protamine added (ng mL^{-1})	Protamine found*, proposed biosensor (ng mL^{-1})	Recovery (%)	Protamine found* by HPLC (ng mL^{-1})
1	50.0	52.1 ± 1.6	104.2	51.0 ± 4.8
	120.0	119.4 ± 2.6	99.5	123.0 ± 7.8
	150.0	148.3 ± 4.3	95.9	143.8 ± 8.3
2	50.0	50.1 ± 1.4	100.3	48.7 ± 3.9
	150.0	148.7 ± 3.5	99.1	152.2 ± 9.5

* Average values of 3 determinations ± standard deviations.

found as 1.0 ng mL^{-1} of protamine.

To assess the precision of the biosensor, protamine determinations were carried out for a set of 10 measurements of 30.0 and 100.0 ng mL^{-1} protamine under the optimal conditions. The results showed a standard deviation of 1.26 and 1.39 for 30.0 and 100.0 ng mL^{-1} protamine respectively.

5. Interference study

In order to investigate the selectivity of method, the influence of common species in biological medium on the protamine determination were tested. For this purpose, the response of the biosensor to several species, including dopamine, glucose, ascorbic acid, urea, K^+, Fe^{3+}, Ca^+, Mg^{2+} (all in 15000 ng mL^{-1}) and protamine in 30.0 ng mL^{-1} were checked under the optimum experimental conditions. As can be seen from Fig. 5, the proposed biosensor demonstrates good selectivity for protamine detection in the presence of large amounts of the potential interfering species. This is due to the fact that protamine has 20-positive charge while the other tested cations have only one, two or three-positive charge and thus have weaker electrostatic interaction with the QDs *vs.* protamine. So, other neutral species or cations with low charge density cannot lead to the significant aggregation of QDs under the experimental condition.

6. Application

The applicability of the biosensor to real sample was tested using blood serum. Standard addition method was used for the determination of protamine in the serum samples. The results are given in Table 1, showed the recovery was acceptable. Also, to check the accuracy of the biosensor, a high performance liquid chromatography (HPLC) was used to measure the protamine in the human serums samples. The protamine concentration (ng mL^{-1}) in the human serums sample were found to be 119.4 ± 6.1 ($n=3$) by the HPLC method whereas it was found as 123.0 ± 7.8 ($n=3$) by the proposed biosensor. Student *t*-test method was used to check the data accuracy. The calculated *t*-value was equal to 1.48, whereas t_{table}(95%,4) was equal to 2.78 ($t_{calculated} < t_{table}$). Thus, the accuracy of the biosensor is acceptable.

Table 2
Comparison of the analytical data of some reported methods for the determination of protamine.

Method	Linear dynamic range	Detection limit	Reference
HPLC	15–100 μg mL^{-1}	15 μg mL^{-1}	Snycerski et al., 1998
Reversed phase HPLC	0.0–114.7 μg mL^{-1}	–	Hvass and Skelbaek-Pedersen, 2005
Electrochemistry	0.4–3 μg mL^{-1}	–	Xiao et al., 2001
Fluorimetry	2.5–17.5 ng mL^{-1}	2.2 ng mL^{-1}	Pang et al., 2014
Fluorimetry	0–0.8 μg mL^{-1}	0.0067 μg mL^{-1}	Zhao et al., 2013
Colorimetry	Up to 0.9 μg mL^{-1}	0.1 μg mL^{-1}	Jena and Raj, 2008
Fluorimetry	1.0–200.0 ng mL^{-1}	0.2 ng mL^{-1}	This work

7. Conclusions

As stated before, protamine is an important pharmaceutical compounds with an anticoagulant effect. Development of a sensitive, rapid and accurate method for measuring protamine is essential. Here a novel biosensor is introduced for quantitative analysis of protamine using glutathione-capped CdTe-QDs. Protamine significantly aggregated the glutathione-capped CdTe-QDs and caused the quenching of the fluorescence. This method is simple, rapid and specific. In addition, low detection limit (0.2 ng mL^{-1}), high selectivity and sensitivity are the most important advantages of the present biosensor. The proposed optical biosensor has been applied successfully to protamine analysis in real samples with satisfactory results. As shown in Table 2, the detection limit and dynamic range of the present biosensor was compared with different methods for measuring protamine.

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