Enhanced Sensitivity of VEGF Detection Using Catalase-Mediated Chemiluminescence Immunoassay Based on CdTe QD/H2O2 System

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Abstract
Background
Since vascular endothelial growth factor (VEGF) is a major regulator of cancer angiogenesis, it is essential to develop a technology for its sensitive detection. Herein, we sensitized a chemiluminescence (CL) immunoassay through the combination of H$_2$O$_2$-sensitive TGA-CdTe quantum dot (QD) as signal transduction, dextran system as a cross-linker to prepare enzyme-labeled antigen and the ultrahigh bioactivity of catalase (CAT) as reporter enzyme.

Results
Under the optimized experimental conditions, the CL-ELISA method can detect VEGF in the excellent linear range of 2–35000 pg mL$^{-1}$, with a detection limit (S/N = 3) of 0.5 pg mL$^{-1}$ which was approximately 10 times lower than the commercial colorimetric immunoassay. This proposed method has been successfully applied to the clinical determination of VEGF in the human serum samples and the results illustrated the excellent correlation with the conventional ELISA method ($R^2 = 0.997$). The suitable recovery rate of the method in the serum ranged from 97–107%, with a relative standard deviation of 1.2–13.4%.

Conclusions
The novel immunoassay proposes a highly sensitive, specific and stable method for very low levels detection of VEGF that can be used in primary diagnosis of tumor. With the well-designed sensing platform, this approach has a broad potential to be applied for quantitative analysis of numerous disease-related protein biomarkers for which antibodies are available.

Background
Accurate detection of tumor markers at low concentration, has significant promise for early diagnosis and therapeutic monitoring (1–3). Vascular endothelial growth factor (VEGF) is a significant regulator of both pathologic and physiologic angiogenesis by activating VEGF-receptor tyrosine kinases in all endothelial cells (4–7). VEGF is a signaling protein that has been applied as an important serum biomarker for a number of human diseases, such as cancer (8–11), rheumatoid arthritis (12), psoriasis (13) and proliferating retinopathy (14). The abnormally rapid growth and division of tumors make it stimulated the overexpression of VEGF due to the supply of more nutrients and oxygen, leading to in
tumor lymphatic vessels induction and cancer metastasis (15). It is estimated that up to 60% of human cancer cells overexpress VEGF to create the essential vascular network to support tumor growth and metastasis (16). A high concentration of VEGF in the tumor tissue and serum of patients who suffer brain tumor was previously reported (17). Increased VEGF concentration is also highly correlated with tumor progression and survival in patients with malignant melanoma (18). According to previous researches, the VEGF level in healthy people is usually less than 100 pg mL$^{-1}$ and clearly increased by the progression of clinical stage (198 pg mL$^{-1}$ in stage I-II; 955 pg mL$^{-1}$ in stage III-IV) in ovarian cancer. Moreover, similar VEGF values were previously reported, indicating overexpression of VEGF in patients with brain tumor (19, 20). Hence, VEGF serum level has important effects as a biomarker on some diseases and subsequent monitoring of treatment.

Avastin, a humanized anti-VEGF antibody, is a potential candidate for the development of antiangiogenic drugs against VEGF (21, 22). Therefore, due to its specificity for VEGF, it is known to be an extremely potent angiogenesis inhibitor (23). It is a challenging and critical task to develop selective and sensitive detection of VEGF in the patients’ whole blood or serum for early diagnosis of disease and efficient therapeutic monitoring strategies.

VEGF$_{165}$ is the most potent pro-angiogenic isoform and is usually overexpressed in a variety of human tumors (24, 25). Therefore, the receptor binding domain (RBD) of VEGF$_{165}$ is selected as the target protein in this work. Tumor markers can be detected by different methods such as radioimmunoassay (26), enzyme-linked immunosorbent assays (ELISA) (27), electrophoretic immunoassay (28), and mass spectrometry-based proteomics (29). ELISA is one of the most common immunoassay methods that is widely used for the detection of different analytes due to its simplicity, rapidity, low cost, robustness, and high-throughput (30). However, the conventional colorimetric ELISA based on horseradish peroxidase (HRP) displays low sensitivity and fails to meet requirements for higher sensitivity application.

Here we improved the detection sensitivity of ELISA by three strategies. At first, catalase (CAT) was used instead of HRP due to its excellent catalytic efficiency. CAT turnover number exceeds any other
enzymatic reaction and is estimated to be approximately $4 \times 10^7$ (31-34), which is almost 240-fold higher than the one for HRP (35). Secondly, increasing the ratio between the enzyme molecule and target analyte at each binding event which can amplify the detection signal and consequently improves the sensitivity of ELISA. Negatively because of steric reasons in conventional ELISA, a ratio of 1:1 for enzyme and detection antibody is usually used (29, 36). We use dextran that can address the problems described above as these molecules exhibit three-dimensional and flexible construction with high loading capacity. Meanwhile, to maintain a high CAT activity of CAT-VEGF conjugate to enhance the detection sensitivity, we proposed the use of the dextran system to achieve an indirect conjugation of the CAT and VEGF. In addition, due to relatively low color intensity, especially at low analyte concentrations, the signal-generation mechanism based on enzymes that catalyze chromogenic substrates (e.g., tetramethylbenzidine) is not suitable. To enhance the detection sensitivity of conventional ELISA, chromogenic substrates may be replaced by more sensitive signal-generation transducers to convert molecular recognition events into detectable outputs, including chemiluminescent substrates, as the third strategy.

Chemiluminescent ELISA (CL-ELISA) is a good alternative method for samples screening with the significant merits of high sensitivity, low noise, no interference from background scattering light, broader linearity, reduced assay time, free of radioactive reagents, simple instrument and ease of use. These advantages of CL-ELISA make it a useful detection system. Up to now, several chemiluminescent immunoassay methods have been established in the clinical detection of tumor markers, such as alpha-fetoprotein (AFP), prostate-specific antigen (PSA), carbohydrate antigen 125 (CA125), and neuron-specific enolase (NSH) (37-40).

Quantum dots (QDs) as a new family of versatile nanoparticle, have shown attractive prospects due to their unique optical properties and effects associated with quantum confinement (41-43). One considerable property of TGA-CdTe QDs is being sensitive to hydrogen peroxide ($H_2O_2$), leading to the QDs CL. We have recently developed a catalase assay method based on the finding that the CL of the TGA-CdTe/$H_2O_2$ system is reduced owing to the consumption of $H_2O_2$ by the catalytic action of CAT.
In the present study, a novel CL based ELISA was developed for the sensitive detection of VEGF, in which H$_2$O$_2$-sensitive TGA capped CdTe QDs were introduced as a CL signal output, avastin (anti-VEGF monoclonal antibody) and CAT-VEGF conjugate as the coating antibody and competitive antigen, respectively. The analytical performances of our proposed method which is assessed on the basis of validation process using real sample demonstrated that the CL-ELISA can be applied for selective detection of VEGF molecules in real samples accurately.

**Methods**

**Chemicals and Materials**

Bovine liver catalase (CAT), bovine serum albumin (BSA) and Dextran T500kD were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The Ni-NTA agarose was provided by Qiagen (Hilden, Germany). Isopropyl-b-thiogalactopyranoside (IPTG) was purchased from Biobasic Inc. (Canada). Commercial anti-VEGF antibody (Avastin) was purchased from Roche (Switzerland, Basel). H$_2$O$_2$ (aqueous solution, 30% w/v) and all other chemicals were obtained from Merck (Darmstadt, Germany). Commercial human VEGF ELISA kit was purchased from Abcam (Cambridge, UK). All the other reagents were analytical grade and used directly without further treatments. Ultrapure water was utilized throughout the experiments. Phosphate-buffered saline (PBS, 0.01 M) was prepared by adding 0.2 g KH$_2$PO$_4$, 2.9 g Na$_2$HPO$_4$ 12H$_2$O, 0.2 g KCl and 8.0 g NaCl into 1000 mL ultrapure water solutions and adjusted to pH 7.0. The washing buffer consisted of 0.05% Tween-20 spiked into phosphate buffer. Blocking buffer for the residual reactive sites was phosphate buffer containing 0.2% BSA.

**Synthesis Of TGA Capped CdTe QDs**

The TGA-capped CdTe QDs with a certain size, concentration and maximum emission wavelength (4 nm, 4.0 x 10$^{-6}$ M and 570 nm respectively), as the optimized conditions for the CdTe QDs/H$_2$O$_2$ CL system, were prepared according to our previous work (44, 45).

**Expression And Purification Of VEGF**

Expression and purification of His-tagged VEGF-RBD (46) (which is hereinafter would be referred as VEGF) were performed as described previously (47) using pET28a expression vector containing VEGF gene in *E. coli* BL21 cells and Ni-NTA agarose column. Protein expression and purification were
evaluated using 12.5% (w/v) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) by the method of Laemmli (48) which then stained by Coomassie Brilliant Blue R250. Excess salt in collected fractions was removed by three times dialyzing against PBS containing 10% (v/v) glycerol by gentle stirring for 12 h at 4 °C. Finally the total VEGF concentration was estimated by the Bradford method, using bovine serum albumin (BSA) as the standard (49).

Preparation of dextran mediated CAT-VEGF conjugate

The CAT-VEGF conjugate was prepared according to the previous report with minor modification (45). In brief, dextran T500kD was activated by 37.5 mg mL$^{-1}$ of periodate in sodium acetate buffer (0.05 M, pH 5.0) at 0 °C for 30 min. Aldehyde production was investigated with 2 mg mL$^{-1}$ of dextran–aldehyde and 2,4-dinitrophenylhydrazine (DNPH, 10%) in 1 M NaOH and formaldehyde was used as a control according to the method of Charbgoo et al (50, 51). The CAT-VEGF conjugate was synthesized by suspending CAT, VEGF and dextran in PBS at a molar ratio of 20: 4: 1. After stirring the mixture in the dark at 10 °C for 72 h, the reactions were stopped by adding 10 µL glycine (2 M). To demonstrate the success of the conjugation reaction, the CAT-VEGF conjugate was characterized by 8% native-PAGE based on the method of Davis (52) which was performed at a constant voltage at 100 V for 120 min at 4 °C and then the gel was stained by Coomassie Brilliant Blue R250.

Gel Filtration

The CAT-VEGF conjugate was separated using Sephadex G-200 (GE Healthcare, Uppsala) gel filtration column equilibrated with 100 mM PB (pH 7.0) at a flow rate of 0.6 mL min$^{-1}$ under the monitoring of $A_{280}$ via an ultraviolet spectrometer. Aliquots of 300 µl of each fraction were collected and the CAT activity was examined via CL-based CAT assay using $H_2O_2$-sensitive TGA-CdTe quantum dots assay (44). The protein components of effective fractions were analyzed by 8% native-PAGE and stained by Coomassie Brilliant Blue.

Optimization Of CL-ELISA

Several physicochemical factors that influenced the chemiluminescent ELISA performance were carefully optimized in this work. In order to evaluate the influence of CAT-VEGF conjugate, direct ELISA was performed as follows: The 96-well plates were first coated with 100 µL of anti-VEGF
monoclonal antibody (1 µg mL$^{-1}$) in PBS (pH 7.0) and incubated overnight at 4 °C. After washing thrice with PBST (PBS buffer containing 0.05% Tween 20, pH 7.0), 300 mL of BSA solution (1.0 mg mL$^{-1}$) was used to block the excess sites of the wells. After 2 h of incubation at 37 °C, the microplate was washed with the same procedure. Subsequently, 100 mL of different dilution of CAT-VEGF conjugate in PBS was added into the wells for 2 h at 37 °C. After washing thrice with PBST and once with PBS, 100 µL of 300 mM H$_2$O$_2$ in 0.01 M PB (pH 7.0) was injected for 1 min. Finally, 100 µL of TGA-CdTe QDs was injected into the well and the CL signals of the TGA-CdTe QDs were measured by using Berthold luminometer (Titertek-Berthold, Sirius L, Pforzheim, Germany). The effect of the enzyme reaction time on substrate was also investigated by the assay procedure described above with constant concentration of CAT-VEGF conjugate in different reaction time of 2-120 sec.

Development Of Direct Competitive CL-ELISA For VEGF
The 96-well microplates were modified with 100 µL of anti-VEGF monoclonal antibody (1 µg mL$^{-1}$) in PBS (pH 7.0) at 4 °C overnight, followed washing three times with PBST then blocked with blocking buffer and washed thrice with PBST after 2 h of incubation at room temperature. Subsequently, 100 µL of VEGF standards was added to a desired final concentration ranging from 0 to 50 ng mL$^{-1}$ by diluting a stock solution with PBS (0.02 M, pH 7.0). Following the incubation for 1 h at room temperature and washing procedure, 100 µL of CAT-VEGF conjugate was added and incubated in the dark. After 1 h at room temperature, the unbounded content was discarded, and the microplates were washed thrice with PBST and once with PBS. Finally, 100 µL of 300 mM H$_2$O$_2$ in 0.01 M PB (pH 7.0) per well was injected to the microplates. After the H$_2$O$_2$ was incubated for 1 min, 100 µL of TGA-CdTe QDs was injected to each well and the CL signals from the TGA-CdTe QDs, related to the VEGF concentrations, were measured by using luminometer.

Validation Analysis Of VEGF Sensor
Human serum was 3-fold diluted with 0.01 M PBS (pH 7.0). Spiked serum samples were prepared by adding the standard VEGF at concentrations of 6.00, 0.220 and 0.020 ng mL$^{-1}$. Samples were analyzed following the assay procedure described above. To compare them, conventional HRP-based
colorimetric immunoassay was conducted by using an ELISA kit according to the operating instructions. The VEGF concentrations were determined using the relevant calibration curves for the CL-ELISA and conventional ELISA assays. All Analyses are always made in triplicate.

Results And Discussion

Synthesis, characterization and optimization of TGA-CdTe-QD

The TGA-CdTe QDs were synthesized and characterized as we previously reported. On the basis of results, we concluded that our TGA-CdTe QDs exhibit uniform size distribution and good optical properties. Similar to the previously reported results, we find that the following experimental conditions give best CL intensity: 800 mM H\textsubscript{2}O\textsubscript{2}, 200 mM NaOH and size around 4 nm TGA-CdTe QDs as synthesized with a concentration of 4.0 × 10\textsuperscript{-6} M (44).

Preparation of CAT-VEGF conjugate

pET28a containing VEGF RBD genes was transformed into E. coli BL21 competent cells followed by IPTG induction. Protein was efficiently expressed and then purified by Ni-NTA resin. After dialysis against PBS, the VEGF concentration was determined and subjected to non-reducing SDS-PAGE analysis. A sharp band of purified expected size (~ 28 kDa) was observed by SDS-PAGE (data not shown).

To prepare enzyme-labeled antigen, a dextran system was designed as a cross-linker to conjugate the VEGF to CAT. The CAT-VEGF conjugate achieved through the formation of amide bond between the -\text{NH}_2 groups of the proteins and the carboxyl group of dextran. Meanwhile, in competitive adsorption on the activated dextran between CAT and VEGF, the CAT possessed advantages in the competition due to the higher concentration compared to VEGF (with the optimized mole ratio of 20: 4: 1 for CAT, VEGF and activated dextran, respectively (data not shown)) which increases the sensitivity of the proposed method. To demonstrate the successful preparation of CAT-VEGF conjugate, native-PAGE was carried out. As shown in Fig. 1a, line 4 contains a conjugate in addition to VEGF and CAT.

In order to separate non-conjugated proteins, the CAT-VEGF conjugate was subjected to gel filtration chromatography using a Sephadex G-200 column and the chromatogram at 280 nm is represented in Fig. 1b. We assumed that the first peak, which definitely has a higher molecular weight, contains CAT-
VEGF conjugate and the second and third peaks contain non-conjugated CAT and VEGF, with a molecular weight of 240 and 28 kDa, respectively. Various experiments were carried out to prove this assumption. The UV-vis spectrum displayed the characteristic absorption peak of the Soret iron (III) heme structure of CAT at 406 nm (53) indicating that the first two peaks contain CAT (Fig. 1b). Meanwhile, CAT activity was examined via CL-based CAT assay using H$_2$O$_2$-sensitive TGA-CdTe QDs (44). Among the three peaks, the first two peaks possessing CAT activity were observed on the chromatogram (Fig. 1b). Protein components of each peak were also analyzed by native-PAGE to monitor the formation and separation of the CAT-VEGF conjugate. As shown in Fig. 1c, the first peak only includes the conjugate, the second peak only contains the CAT and the third peak only holds the VEGF. Furthermore, all three peaks were subjected to direct ELISA for VEGF detection. As shown in Fig. 1d, peak 1 and peak 3 contain VEGF. These results demonstrated that CAT and VEGF were covalently attached to the dextran and purified from non-conjugated proteins successfully.

**Optimization of the developed CL assay**

Experimental conditions including the concentration of competitive antigen (CAT-VEGF conjugate), H$_2$O$_2$ and reaction time of CAT, as the most important factors, were investigated to perform developed method under the optimized conditions and improve the immunoassay sensitivity. The concentration of CAT-VEGF conjugate was optimized to obtain lower CL response for the positive and higher CL signal for the control sample. Different dilutions of CAT-VEGF conjugate were evaluated by direct ELISA followed by measuring the corresponding CL intensities of our developed system. The results in Fig. 2a suggest that the CL intensity decreased with increasing the CAT-VEGF conjugate concentration and then reached a minimum value when the OD$_{280}$ was almost 0.3. Therefore, this dilution of CAT-VEGF conjugate was selected as the optimal concentration in subsequent experiments (VEGF and dextran alone, as a control, had no effect on CL intensity).

The effect of the enzyme reaction time on the detection sensitivity of the immunoassay experiments obtained by direct ELISA is shown in Fig. 2b. The CL signal decreased greatly as the enzyme reaction time extended, and then reached the plateau when the time prolonged to 2 min. Thus, the enzyme reaction time of 1 min was necessary to achieve the highest efficiency for the consumption of H$_2$O$_2$.
by CAT. Based on our previous reports, the alteration in CL of the TGA-CdTe QDs has a good relationship with the concentration of H$_2$O$_2$ ranging from 0 to 800 mM demonstrating that the CL of the synthetic TGA-CdTe QDs was extremely sensitive to H$_2$O$_2$ concentration in solution (44). To obtain a stable CL signal that is also sensitive enough to H$_2$O$_2$ reduction, we designated 300 mM H$_2$O$_2$ as the optimal concentration in the subsequent experiments. Under the optimized concentration of H$_2$O$_2$, the CL intensity of the QDs would be decreased with the increased CAT concentration due to the decomposition of H$_2$O$_2$. The previous results indicate that a low amount of CAT was able to cause significant changes in H$_2$O$_2$ concentration in order to generate remarkable CL signal fluctuations and subsequently generates the ultrahigh sensitivity of the CL immunoassay.

The following experimental conditions are found to display best results: a capture antibody concentration of 1.0 µg mL$^{-1}$; H$_2$O$_2$ concentration of 300 mM, CAT-VEGF conjugate at OD$_{280}$ = 0.3 and enzyme reaction time of 1 min.

**Development of direct competitive CL-ELISA based on H$_2$O$_2$-induced CL of QDs**

The schematic illustration of the proposed competitive CL-ELISA is outlined in Scheme 1. The CAT-VEGF conjugate was used as a competitive antigen, and the QD/H$_2$O$_2$ system was used as the signal transduction of ELISA. The CAT-VEGF conjugate was captured by the anti-VEGF monoclonal antibody pre-coated on the 96-well plate while detecting negative samples. CAT on the conjugate would consume H$_2$O$_2$. The remaining H$_2$O$_2$ could trigger QDs, thereby induces the CL signal. Conversely, less amount of CAT-VEGF conjugate was captured when dealing with VEGF-positive samples, hence a low amount of H$_2$O$_2$ was consumed. More H$_2$O$_2$ led to a stronger CL of TGA-CdTe QDs, and a higher CL signal was obtained. Therefore, recording the change in CL signals would permit the detection of analytes (Scheme 1).

**VEGF detection using CL-ELISA**

Under the optimized conditions, a direct competitive CL-ELISA curve was established. The CL was plotted against the logarithm of various concentrations of the VEGF standard solution (0–50 ng mL$^{-1}$).
The calibration curve of the developed CL-ELISA exhibited a superior linear range from 2-35000 pg mL$^{-1}$ with a reliable correlation coefficient ($R^2 = 0.991$). The regression equation is represented by $y = 193321x + 1.868 \times 10^6$, where $y$ is the CL and $x$ is the VEGF concentration. In this calibration curve, the detection limit defined as the lowest assayed concentration of analyte which yields a signal higher than three times the standard deviation of the blank (54) is 0.5 pg mL$^{-1}$. This value is 10-fold lower than the conventional HRP-based ELISA (5 pg mL$^{-1}$) and possesses the highest sensitivity and low background signal. These results validate the utilization of CL-ELISA for the identification of serum VEGF at low concentrations. The excellently low LOD is attributed to the high efficiency of the CAT, dextran-mediated conjugation and the use of CL signal of TGA-CdTe QDs.

**Validation studies**

The specificity was determined by evaluating the cross-reaction of the proposed method with 10 common interfering substances in human serum, including PDGF, TGFβ, HAS, BMP, IL-12, IL-2, IL-1β, IFNγ, IGF-1 and IgG4. In comparison with the negative control, a higher CL was observed when the target VEGF concentration was 50 ng mL$^{-1}$ while no significant differences of response signals were observed when other non-specific proteins were detected at the same concentrations (Fig. 4). This finding demonstrates that our strategy has a high selectivity toward the target and those interfering species would not affect the accuracy of results in the clinical diagnosis determination.

To evaluate the accuracy and precision of the proposed detection system, recovery assay was conducted in three replicates of VEGF-spiked serum samples at theoretical concentrations of 6.00, 0.220 and 0.020 ng mL$^{-1}$ and the increase in the measured concentration of VEGF was determined by comparison to normal serum. Resulting levels in the clinical specimens were then quantified using the calibration curve. The calculated average recovery value was in the range of 76.5–96.7% with the relative standard derivation of 4.88–16.4%, thereby indicates good accuracy, high reproducibility and precision of the developed CL-ELISA for quantitative detection of VEGF in actual serum samples. The results also indicated no interference of complex matrices on the developed strategy.
The storage stability of the developed CL immunoassay was studied over a period of one month (data not shown). Prepared devices were kept at 4 °C under dark condition and then were used to measure the current response after one month. We see no obvious variations in the detection system. Therefore, the stability was confirmed to be acceptable.

Finally, the reliability of analytical performance and practical value of the developed method was established by comparing the results with those of colorimetric immunoassay kit obtained from blindly detecting five real serum samples. Each sample was analyzed in triplicate and levels were determined by using the calibration curve. The data obtained from our method were compared with the result by conventional ELISA method. The concentrations of VEGF in the serum of normal individuals were relatively low compared to the breast, uterus and brain cancers patients' serum samples. The relationship between the two methods was assessed by linear regression analysis and the result was shown in Fig. 5. The two methods exhibited good agreement with a highly significant correlation value \( Y = 0.9988x - 6.212, R^2 = 0.997 \). The x-axis represented the concentrations of VEGF detected by CL-ELISA, and the y-axis was the concentrations of VEGF detected by colorimetric ELISA. The results indicated high consistency which reveals that the developed method could be reliable and well suited as a quantitative tool for the analysis of VEGF and even other tumor biomarkers in clinical diagnosis.

**Conclusion**

In this study, we demonstrated a novel convenient CL-sensing protocol to detect VEGF by using a competitive immuno reaction strategy. Through the incorporation of three signal amplification factors, including \( \text{H}_2\text{O}_2 \)-sensitive TGA-CdTe QDs as a CL signal output, dextran-mediated conjugation and ultrahigh catalytic activity of CAT to \( \text{H}_2\text{O}_2 \), the proposed immunoassay exhibited high sensitivity for detection of VEGF with an LOD value at 0.5 pg mL\(^{-1}\), which was 10-fold lower than the conventional ELISA. The analytical performances of our developed method were evaluated in term of accuracy, precision, specificity, and practicability using VEGF-spiked and patient serum sample. The results demonstrated that our proposed CL-ELISA can be applied for the ultrasensitive detection of VEGF in actual serum samples. Furthermore, the results obtained from the proposed method showed a
significant correlation with the findings from the confirmatory method. Taking into account of the low detection limit, wide linear range, high reproducibility, excellent specificity, good accuracy, suitable practicability and stability of the immuno sensor, it can be attempted to use for highly sensitive detection of VEGF and numerous analytes, for which antibodies are available, in clinical diagnostics.

Abbreviations

VEGF: vascular endothelial growth factor; CL: chemiluminescence; QD: quantum dot; CAT: catalase; ELISA: Enzyme-linked immunosorbent assays; RBD: receptor binding domain; HRP: horseradish peroxidase; AFP: alpha-fetoprotein; PSA: prostate-specific antigen; CA125: carbohydrate antigen 125; NSH: neuron-specific enolase; IPTG: Isopropyl-b-thiogalactopyranoside; PBS: Phosphate-buffered saline; SDS-PAGE: sulfate polyacrylamide gel electrophoresis; BSA: bovine serum albumin; DNPH: 2,4-dinitrophenylhydrazine.

Declarations

Authors’ contributions

FG carried out experiments, analyzed data and wrote the manuscript, HR participated and assisted in data analysis, MS assisted in the synthesis of the nanoparticle, KK and MM gave technical support during in this project, RHS supervised entire project and involved in the designing of all experiments and revised the manuscript. All authors approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

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Figures
(a) Native-PAGE analysis of dextran mediated conjugation of CAT with VEGF. Lanes 1, 2 and 3 contain dextran, VEGF and CAT as control, respectively; Lane 4, constitutes the conjugate of CAT-VEGF after incubation time. (b) Gel filtration chromatogram using a Sephadex G-200 column. Elution was performed with PB (pH 7.0) at a flow rate of 0.6 mL min-1, and the absorbance of each fraction (300 µl/fraction) was monitored at 280 nm. Inset plots are CAT activity of each peak which was evaluated via CL-based CAT assay using H2O2-sensitive TGA-CdTe QDs. (c) Native-PAGE analysis of CAT-VEGF conjugate after purification. Lanes 1 and 3 contain VEGF and CAT as control, respectively; Lanes 2, 4 and 5 correspond to peak 3, 2 and 1, respectively. (d) Colorimetric ELISA results for VEGF detection of each peak (Inset: the corresponding photographs)
Parameter optimization of the proposed CL-ELISA. (a) CAT-VEGF conjugate concentration. (b) Enzyme reaction time between CAT and H2O2. The error bars represent the standard deviation of three parallel measurements. Data were fitted to a nonlinear regression of
A quantitative immunoassay of VEGF using the developed CL-ELISA. The inset shows a dynamic linear range of VEGF concentrations from 0.002-35 ng mL⁻¹. The error bars represent the standard deviation of three measurements.
Specificity of the proposed CL-ELISA assay for VEGF detection over other interfering substance. Each independent experiment was repeated 3 times. VEGF, PDGF, TGFβ, HAS, BMP, IL-12, IL-2, IL-1β, IFNγ, IGF-1 and IgG4 samples were all prepared in concentration of 50 ng mL⁻¹. A negative control test was performed by adding PBS
Figure 5

Correlation of the CL-ELISA and conventional colorimetric ELISA results for VEGF detection in 5 serum samples \((y = 0.9988x - 6.212, R^2 = 0.997)\). Each point represents an average of three replicates.
Scheme 1 Schematic principle of the CL-ELISA based on H2O2-sensitive QDs for the detection of VEGF. Anti-VEGF monoclonal antibody was employed as the capture antibodies. Dextran was designed as a bridge to connect the VEGF and CAT, and H2O2-sensitive TGA-CdTe QDs were used as CL signal output.