JAAS

Cite this: *J. Anal. At. Spectrom.*, 2011, **26**, 2493

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PAPER

Inductively coupled plasma mass spectrometry for determination of total urinary protein with CdTe quantum dots label

Yurong Tang,[a] Xue Jiao,[a] Rui Liu,[a] Li Wu,[b] Lan Wu,[b] Xiandeng Hou[ab] and Yi Lv*[a]

Received 24th July 2011, Accepted 15th September 2011
DOI: 10.1039/c1ja10213c

In the present work, a novel methodology for sensitive quantification of human total urinary protein by using CdTe quantum dots label and inductively coupled plasma mass spectrometry (ICP-MS) was developed. It combined the intrinsically high sensitivity of elemental mass spectrometric analysis with the remarkable quantity of metallic ions from the nanoparticles label. Through the direct conjugation of CdTe quantum dots and proteins, the ICP-MS signal intensity of cadmium is proportional to the concentration of proteins. The optimization of bio-conjugation parameters was investigated and discussed in detail. The proposed method featured a low detection limit (0.008 μg mL^{-1} for HSA) and wide linear range response over 4 orders of magnitude. Moreover, there are very little interferences from common inorganic ions and other coexisting compounds. The developed method was successfully applied for detecting total protein in 50 human urine samples and the results were in good agreement with those reported by the hospital, which indicated a great potential for routine clinical application.

1 Introduction

Quantitative analysis of proteins is critically important in biomedical research, clinical diagnosis, and laboratory practice biology, since the quantity of proteins or changes in their abundance directly reflects the status and changes of a biological system. Traditionally, routine analysis for the determination of total proteins involves several colorimetric assays, such as Lowry,[1] Bradford,[2,3] Bromocresol Green (BCG)[4] and Bicinchoninic Acid (BCA)[5] assay. However, the above-mentioned methods have limitations in terms of sensitivity and dynamic linear range, implying that quantitative analysis of proteins is still a challenging issue. Therefore, a great number of new protocols have been frequently attempted, such as those involving spectrophotometry,[6,7] spectrofluorimetry,[8,9] resonance light scattering (RLS),[10,11] chemiluminescence,[12] electrochemistry,[13] surface-enhanced Raman scattering (SERS)[14,15] and mass spectrometry (MS).[16]

Recently, the combination of inductively coupled plasma mass spectrometry (ICP-MS) and elemental tagging methods has been attempted for quantitative proteomics.[17] ICP-MS tends to be a sensitive readout method for elemental tags, due to its low detection limits, low matrix effects, large dynamic range and high spectral resolution for elements and isotopes.[18,19] Besides metal ion labeling,[20,21] nanoparticles labeling is an intriguing alternative, because of their high signal to noise ratio, low cost and long lifetime.[22] Meanwhile, the use of ICP-MS as a readout method does not require nanomaterial reporters to possess optical, electric, electrochemical, magnetic or any other special properties since ions from the atoms of the elements are directly detected by this instrument. In principle, by selecting nanoparticles instead of metal ions as a tag for biomolecules, ICP-MS can indirectly detect biomolecules with extremely high sensitivity, due to large quantities of detectable atoms in each nanoparticle tag (for instance, 3.1×10^4 atoms in a 10 nm Au nanoparticle).[23] Due to these outstanding properties, ICP-MS is expected to be a promising tool in proteomics studies.[24]

Quantum dots (QDs), as novel semiconductor materials, have been applied as one of the most effective nanomaterials in bioanalysis.[25–28] They are water-soluble and easy to conjugate with biomolecules.[29,30] The application of QDs was mainly focused on fluorescent analysis[31–33] and electrochemical stripping analysis,[34,35] thanks to their attractive fluorescent and redox properties. Additionally, QDs are also potentially ideal labels for detecting important biomolecules at low concentrations, due to the large number of atoms per conjugate. However, so far, little work has been reported on the detection method based on QDs' intrinsic elemental composition.[36]

Herein, a highly sensitive QDs-based assay with ICP-MS is presented for the determination of urinary total protein, which is one of most common indicators of nephropathy in patients suffering from diabetes and hypertension.[37] It combines the intrinsically high sensitivity of elemental mass spectrometric analysis with the remarkable quantity of metallic ions from the nanoparticles label. Through the direct conjugation of CdTe quantum dots and proteins, the ICP-MS signal intensity of

[a]*Key Laboratory of Green Chemistry & Technology, Ministry of Education, College of Chemistry, Sichuan University, Chengdu, Sichuan, 610064, China. E-mail: lvy@scu.edu.cn; Fax: +86 28 8541 2798; Tel: +86 28 8541 2798*
[b]*Analytical & Testing Center, Sichuan University, Chengdu, Sichuan, 610064, China*

cadmium is proportional to the concentration of proteins. The experimental conditions, including optimization of bio-conjugated parameters, analytical performance, interferences study and clinical human urine sample analysis, were investigated and discussed in detail.

2 Experimental section

2.1 Instrumentation

An X Series ICP-MS (Thermo Electron Co., Winsford, Cheshire, UK) was used throughout this work. A standard glass concentric nebulizer and a standard glass conical impact bead spray chamber were employed with an uptake rate of around 1 mL min^{-1}. The flow rates of the water stream and cadmium diluted solution were both 0.5 mL min^{-1} through a Y-shaped pipeline. Prior to analysis, the experimental parameters were tuned using 1% HNO_3 solution containing 10 ng mL^{-1} In. The optimized parameters and details of the instrumental settings are listed in Table 1.

The morphologies of the QDs were observed with high-resolution transmission electron microscopy (FEI, Tecnai G2 F20 S-Twin). The QDs solution was dropped onto a carbon-coated copper grid, after which the grid was allowed to dry at room temperature. Absorption spectra and fluorescence spectra were obtained using a HITACHI U-2910 spectrophotometer and a HITACHI F-7000 fluorescence spectrophotometer, respectively. A EUTECH CyberScan pH510 pH meter was employed for pH measurements.

2.2 Reagents

Pure water (18.2 MΩ cm^{-1}) from a water purification system (ULUPURE, Chengdu, China) was used in this work. Polystyrene 96-well microtiter plates (468667, NUNC, Denmark) were used as substrate. Human serum albumin (HSA), Transferrin (Tf) and immunoglobulin G (IgG) standard proteins were purchased from Bejing Solarbio Science & Technology Co. Ltd. (Beijing, China). Stock standard solution of protein powders in pure water, with a concentration of 10 mg mL^{-1} was stocked at 4 °C, being diluted in carbonate/bicarbonate buffer (0.05 M, pH = 9.6) only prior to use. 3-Mercaptopropanoic acid (MPA) was purchased from Alfa Aesar Co. Ltd. (Beijing, China). Unless otherwise stated, all the reagents used in this study were at least of analytical grade and were obtained from Changzheng Chemical Reagent Co. Ltd. (Chengdu, China).

Table 1 Working parameters of ICP-MS

Parameters	Values
RF power/W	1200
Cool gas flow/L min^{-1}	13
Auxiliary gas flow/L min^{-1}	0.8
Nebulizer gas flow/L min^{-1}	0.85
Sample uptake rate/mL min^{-1}	1
Torch	Shield torch
Resolution	Standard
Dwell time/ms	10
Channels	3
Sweeps	100

2.3 Preparation of QDs

MPA-modified CdTe QDs were synthesized by the hydrothermal method mainly according to ref. 38. In brief, 256 mg of potassium borohydride and 56.4 mg of tellurium powder (4 : 1 molar ratio) were added into a flask (25 mL), and then 10 mL of ultrapure water was added. The solution was stirred at room temperature, until a color change occurred from violet to colorless (~4 h). During the reaction, a small outlet connected to the flask was kept open to release the produced hydrogen, and the reaction system was free of oxygen because of the hydrogen pressure. Subsequently, the freshly prepared KHTe solution (5.0 mM, 10 mL) was added to N_2-saturated $CdCl_2$ solution (12.5 mM, 100 mL) at pH 9.2 in the presence of MPA as a stabilizer. The molar ratio of Cd^{2+} : MPA : KHTe was 1 : 2.4 : 0.4. A yellow CdTe colloidal solution was obtained after vigorous stirring and then refluxed for 3 h. CdTe QDs with different sizes were prepared by varying the reflux time. The obtained CdTe QDs were precipitated with ethanol and separated by centrifugation at 4000 r min^{-1} for 15 min. After two cycles, the purified QDs were redissolved and settled as the stock solution (72 μM, calculated according to equation[39]).

Before experiments, MPA-stabilized CdTe QDs solution used for labeling proteins was prepared by diluting 300 μL stock CdTe QDs solution and 60 μL MPA stabilizer into water to obtain 10.0 mL incubating solution (2.16 μM), the pH value of which was adjusted to 4.6 by NaOH (1 M).

2.4 Procedures

The assay was performed in a polystyrene 96-well microtiter plate. The schematic diagram was shown in Fig. 1. 200 μL of diluted protein standard solution or urine samples in carbonate/bicarbonate buffer were incubated in microtiter plates at 4 °C overnight. Protein steadily attached on the solid polystyrene substrate *via* physical adsorption between hydrophobic groups of the biomolecules and polystyrene. The unbound protein was washed off three times with 350 μL washing buffer (0.01 M phosphate buffer saline (PBS) with 0.05% (v/v) Tween 20, pH 7.4). Then, the proteins on the wells were incubated with 200 μL MPA-stabilized CdTe solution (pH 4.6) for 3 h to ensure a thorough reaction of the protein with the CdTe QDs. Unconjugated CdTe particles were removed by 350 μL washing buffer three times. Finally, the QDs–protein conjugates were dissolved in 250 μL 5% (v/v) nitric acid solution for 20 min and diluted to 4 mL with 1% (v/v) nitric acid solution. The Cd signals obtained from the diluted solution were recorded by ICP-MS for analyte quantification.

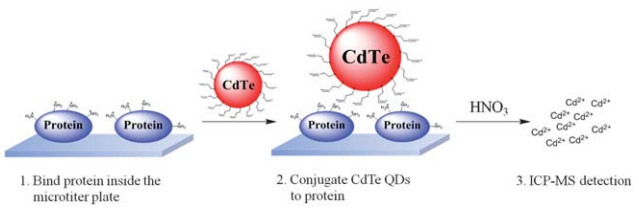

Fig. 1 Schematic diagram of protein determination.

2.5 Urine samples

Urine samples were obtained from patients (Chengdu 7th People's Hospital, Chengdu, China). The total protein concentration (mg L^{-1}) of the urine samples was determined using the hospital reference method (Pyrogallol red–molybdate complex method). Subsequently, the samples were treated with a preservative[40] and frozen for further use. Prior to urinary analysis using this proposed method, the urine samples were thawed at room temperature, centrifuged at 5000 rpm for 10 min and diluted with the carbonate/bicarbonate buffer 100 times to reduce the interference from the matrix and other biomolecules in urine.

3 Results and discussion

3.1 Characterization of CdTe QDs

The TEM image of the prepared CdTe QDs is shown in Fig. 2a. As indicated, the average size of MPA-stabilized CdTe nanoparticles is about 4.5 nm and the size distribution is relatively uniform. In addition, the Fourier transform electron diffraction (FT-ED) pattern indicates that the CdTe QDs appear as spherical particles with a crystalline structure (inset of Fig. 2a).

Fig. 2b presents the change in both the absorption and fluorescence spectra when CdTe QDs are labeled with protein. The absorption spectrum of QDs–protein bioconjugates is flatter than that of the free QDs. At the same time, the absolute absorbance value increases. The emission peak of the unconjugated QDs is at 559 nm; however, the emission peak of QDs–protein undergoes a red shift (566 nm), but the intrinsic spectral width is unchanged. The changes in the absorption and fluorescence spectra may be caused by the shortened distance between QDs due to the attraction between QDs and proteins. The formation of an arrangement of their molecules and the complexes gradually augments with excess QDs, the distance between QDs becomes shorter and shorter, which enhances the dipole–dipole interactions of the QDs and hence cause a larger Stokes' loss.[41,42]

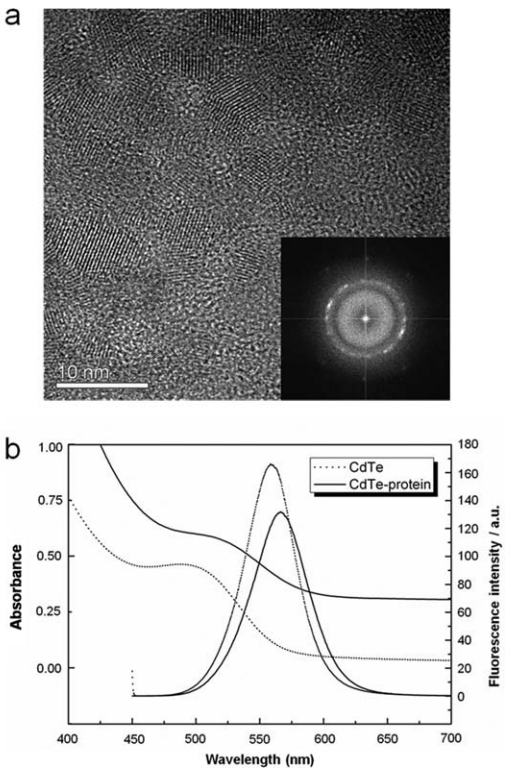

Fig. 2 Characterization of CdTe QDs. (a) TEM image of MPA-stabilized CdTe QDs (inset, the FT-ED pattern). (b) Absorption and fluorescence spectra of CdTe nanoparticles solution (dashed lines) and CdTe–protein solution (solid lines). Excitation wavelength is 370 nm. The protein has no absorption at 370 nm and does not fluoresce under 370 nm excitation.

3.2 Optimization of bioconjugation parameters

The effect of MPA-stabilized CdTe QDs and protein bioconjugation parameters including pH, ionic strength, time, the MPA concentration and CdTe concentration were optimized using 10 μg mL^{-1} HSA solution.

3.2.1 Effect of pH value. The MPA-stabilized CdTe QDs have been proven to have the ability to adsorb proteins and the adsorption strength is mainly dependent on the pH value.[43] As shown in Fig. 3a, we studied the effect of pH ranging from 3 to 11 for the assay performance. High efficiency of adsorption was obtained for pH values between 4.6 and 5.0, and the signal intensities of the system began to decrease sharply after pH 5.0. Therefore, pH 4.6 was chosen as the best pH condition for the effective formation of the CdTe QDs–protein adducts.

The pH effect on adsorption can be explained by the properties of the protein and the nanoparticle surface. The MPA-stabilized CdTe nanoparticles surface carries a negative charge introduced by MPA ions giving necessary colloidal stability to the nanoparticles and preventing aggregation, which also can form a linkage with the protein. When the pH of the QDs buffer is lower than the pI value of most human proteins (*i.e.* HSA 4.7,

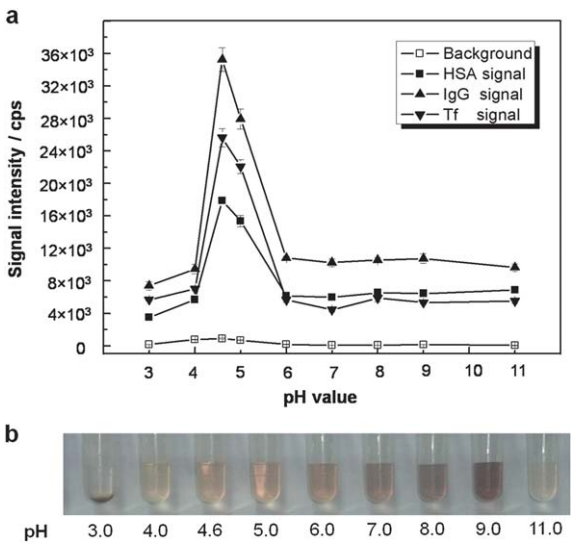

Fig. 3 (a) Dependence of ICP-MS signal intensity upon pH value for the QDs–protein bioconjugation. HSA, IgG, Tf: 10 μg mL^{-1}; CdTe QD concentration: 2.16 μM; MPA concentration: 0.6% (v/v); time: 3 h. (b) Effect of pH on the stability of CdTe QDs.

IgG 8.5, Tf 9.6), most proteins carry a net positive charge and adsorb efficiently to the CdTe nanoparticles surface. In addition, entropic effects may have influence on protein adsorption to nanoparticles.[44] At low pH, proteins were likely to expose protein hydrophobic moieties leading to hydrophobic interactions at the particle surface. On the other hand, at pH values lower than the pK_a of MPA (4.32), the particle surface charge approaches zero, evidently reducing protein adsorption. As shown in Fig. 3b, CdTe nanoparticles aggregation was observed at pH values lower than 4.0. Meanwhile, at pH values higher than 5.0, the luminescence of CdTe QDs begins to decrease at higher NaOH concentration.[45] To summarize, proteins at this pH were most likely conjugated to QD complexes by electrostatic forces and hydrophobic interactions,[46] or combined with other interactions such as nonspecific adsorption and hydrogen bonding.[47,48] Since there are several factors that may affect the CdTe QDs conjugation to proteins, further studies on the mechanisms of CdTe QDs–protein interactions are required.

3.2.2 Effect of ionic strength. We investigated the effect of NaCl content on the ICP-MS signal intensity. As shown in Fig. 4, the signal intensity remained constant with increasing salt concentration up to 0.1 M. That is, low ionic strength has no effect on the assay because the salt concentration of urine is less than 0.1 M.

3.2.3 Effect of reaction time. We studied the appropriate time for the interaction between MPA-stabilized CdTe QDs and proteins. As shown in Fig. 5, the ICP-MS signal increased rapidly with increasing incubation time from 0 to 3 h and then leveled off after 3 h. In the view of time efficiency, an incubating time of 3 h was adopted for CdTe QDs-based interaction. The experimental results indicated that, as well as electrostatic forces, there are some other interactions such as hydrophobic interactions and hydrogen interactions driving the interaction between QDs and proteins, and these require a greater reaction time.

3.2.4 Effect of the MPA concentration. MPA was added into the CdTe solution, not only for colloidal stability but also to contribute sufficient carboxylic negative ions for the system.[38]

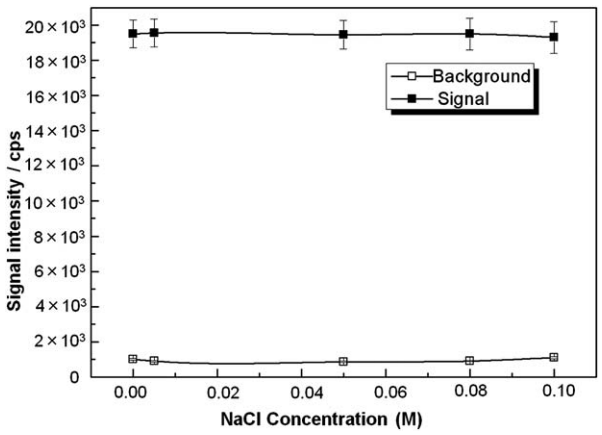

Fig. 4 Dependence of ICP-MS signal intensity upon ionic strength for the QDs–protein bioconjugation. HSA: 10 μg mL^{-1}; CdTe QD concentration: 2.16 μM; MPA concentration: 0.6% (v/v); time: 3 h; pH value: 4.6.

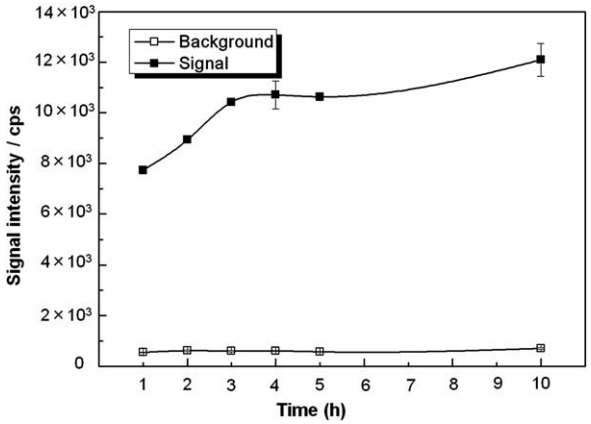

Fig. 5 Dependence of ICP-MS signal intensity upon on the QDs–protein bioconjugation time. HSA: 10 μg mL^{-1}; CdTe QD concentration: 2.16 μM; MPA concentration: 0.6% (v/v); pH value: 4.6.

Optimization of the MPA concentration in the incubating solution was carried out to achieve better bioconjugation. The results in Fig. 6 showed the efficiency of adsorption was enhanced with increasing MPA concentration from 0.30 to 0.60% (v/v), and a maximum was obtained when MPA concentration was between 0.50% (v/v) and 0.60% (v/v). Above 0.60% (v/v), the signal became low. Therefore, a MPA concentration of 0.60% was selected for further study.

With increasing MPA concentration, the conjugation between QDs and proteins becomes stronger due to the fact that addition of MPA not only prevents aggregation between QDs, but also provides more –SCH_2COOH on the surface of the QDs through S–Cd covalent interaction between the free MPA and the Cd atom, and further promotes the adsorption between the protein and QDs' surface. The signal became low at high MPA concentrations, which may be due to the excess free MPA in the colloidal solution which could directly conjugate to proteins.

3.2.5 Effect of the concentration of CdTe solutions. In our study, the influence of the concentration of CdTe QDs was

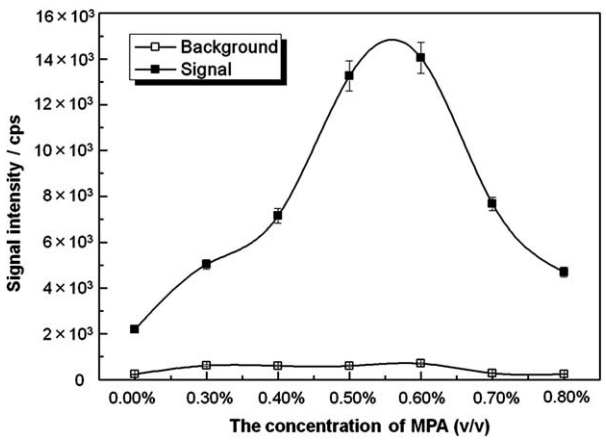

Fig. 6 Dependence of ICP-MS signal intensity upon MPA concentration for the QDs–protein bioconjugation. HSA: 10 μg mL^{-1}; CdTe QD concentration: 2.16 μM; time: 3 h; pH value: 4.6.

investigated. The MPA-stabilized CdTe QDs solution was diluted by stock solution and the final concentration of incubating solution was investigated in the range of 0.72 μM to 4.32 μM. As shown in Fig. 7, the ICP-MS signal enhanced nearly linearly with increasing QD concentration from 0.72 to 2.16 μM. However, there was no further signal increase upon addition of excess CdTe QDs because the bioconjugation between QDs and proteins had reached saturation. Thus, a QD concentration of 2.16 μM was selected for further study.

3.2.6 Effect of the size of CdTe QDs. In our work, the effect of particle size was studied. Firstly, CdTe QDs with different sizes of 1.5, 2.3, 2.7, 2.8 and 3.3 nm, calculated from their luminescence peaks at 527, 542, 570, 594 and 631 nm, were prepared *via* refluxing for 10 min, 30 min, 3 h, 7.5 h, 29 h, respectively. As shown in Fig. 8, the sensitivity and signal response did not vary with the change in particle size. Considering the stability of the QDs solution, the CdTe QDs which were refluxed for 3 h were used throughout this study.

3.3 Different kinds of dissociation solution

The dissociation solution is a key factor affecting the detection sensitivity of the method. In order to completely dissociate the QDs labeled proteins and release Cd quantitatively, the effect of different solutions (0.1% HNO_3, 1% HNO_3, 5% HNO_3, 5% HCl, 5% H_2SO_4, 5% aqua regia) was investigated. The best signal to noise ratio and effective dissociation was obtained while using 5% HNO_3. Consequently, 5% HNO_3 was selected as the dissociation solution in our study.

3.4 Stability of the MPA-stabilized CdTe QDs

The stability of the MPA-stabilized CdTe QDs solution was investigated. Fig. 9 indicates that the absorption ability of MPA-stabilized CdTe QDs is unchanged after 14 days. Because of the good stability of CdTe QDs, the reagent does not need to be prepared freshly every day. These MPA-stabilized CdTe QDs exhibited great suitability for practical application, thanks to their good chemical stability day after day.

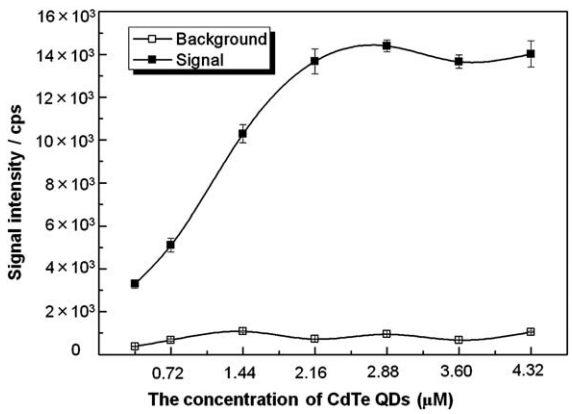

Fig. 7 Dependence of ICP-MS signal intensity upon CdTe QD concentration for the QDs–protein bioconjugation. HSA: 10 μg mL^{-1}; MPA concentration 0.6% (v/v); time: 3 h; pH value: 4.6.

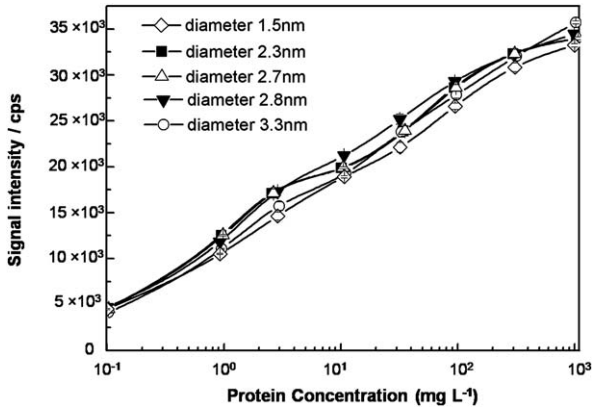

Fig. 8 The response of the HSA assay using five different sizes of QDs.

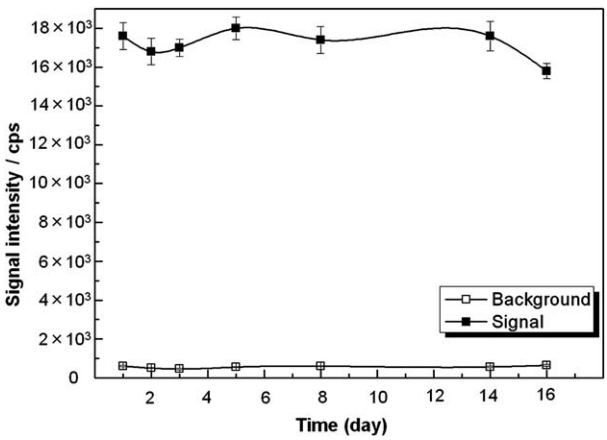

Fig. 9 The stability of MPA-stabilized CdTe QDs solution.

3.5 Calibration curve and sensitivity

The calibration graphs for HSA, Tf and IgG were constructed by following the standard assay procedure. As shown in Fig. 10, the linear ranges for HSA, Tf and IgG are all 0.1–1000.0 μg mL^{-1}, the correlation equations were $Y = 7.6E3\ \lg[HSA] + 1.2E4$, $Y = 1.1E4\ \lg[Tf] + 1.1E4$ and $Y = 1.5E4\ \lg[IgG] + 1.2E4$, with correlation coefficients of $r = 0.9992$, 0.9990 and 0.9988 ($n = 8$), respectively. The limits of detection (3σ) are correspondingly 0.008, 0.02 and 0.03μg mL^{-1} (*i.e.*, 0.12 nM, 0.3 nM, 0.2 nM). For a single experiment with triplicate samples, average coefficients of variation (relative standard deviation, RSD) for 10 μg mL^{-1} HSA were 3.6% for within-batch (intra-assay) or 5.4% for between-batch (inter-assay), which fulfilled the requirements of reliable results. A comparison of the LODs obtained in this work with those obtained by several other approaches was shown in Table 2.[4,8,10,14–16,31,48–50] It is worth mentioning that much lower detection limits and wider dynamic linear ranges were obtained by ICP-MS.

3.6 Influence of foreign coexisting substances

The influence of various foreign species on the determination of protein in urine was investigated. We tested this by premixing HSA with interfering substances, including amino acids, salts, carbohydrates, surfactants and other compounds commonly

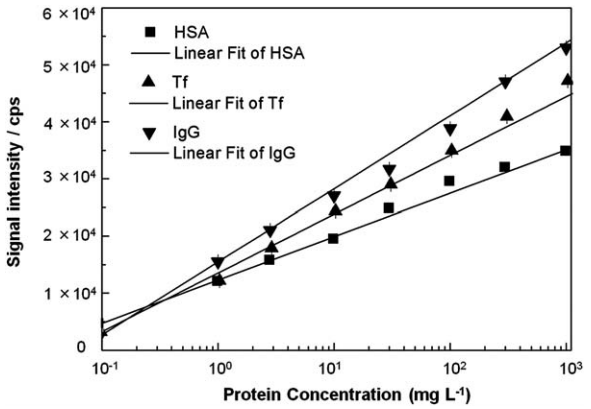

Fig. 10 The relationship between the concentration of three different proteins and ICP-MS signal intensity.

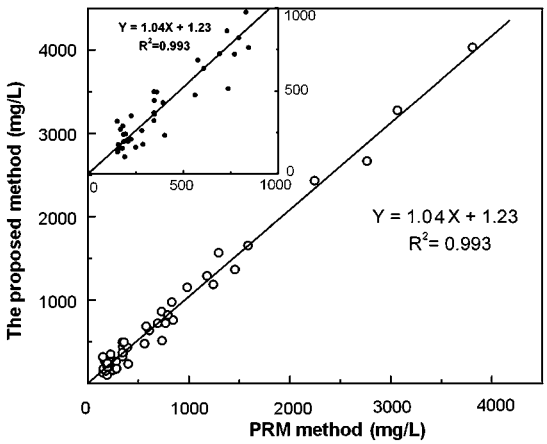

Fig. 11 Correlation plot for measurements performed with this proposed method and a hospital reference method (Pyrogallol red–molybdate complex method) on urine samples. The inset figure represents the total protein concentration of urine samples is in the range of 0–1000 mg mL^{-1}.

Table 2 Comparison of detection limits reported in literature for total protein determination

Label	Detection	LOD/ ng mL^{-1}	Ref
CdTe QDs	ICP-MS	8	This work
Eu chelate complex	SDS-PAGE, LA-ICPMS	100	49
AuNPs	CE-ICPMS	0.033	16
NanoOrange reagent	Spectrofluorimetry	20	8
CBQCA reagent	Spectrofluorimetry	10	50
Bromecresol Green reagent	Spectrophotometry	50 000	4
FRV dye	RLS	25	10
AgNPs	SERS	500	14
Coomassie \Brilliant Blue	SERS	1	15
ZnS nanoparticles	Spectrofluorimetry	15 000, 4.4	51,52
CdTe QDs	PAGE	100	31

Table 3 Tests for the interference of coexisting substances

Coexisting substance	Coexisting conc./μg mL^{-1}	Relative error (%)
Leucine	100	−2.4
Cysteine	100	−5.0
Serine	100	−4.2
Phenylalanine	100	−4.9
Arginine	100	−2.7
L-hydroxyproline	100	0.9
Cholesterol	100	−1.2
Citric acid	100	2.3
Creatinine	100	2.0
Urea	100	1.6
Glucose	100	0.9
Sodium dodecylsulphate (SDS)	100	1.4
NH_4Ac	100	−1.5
KCl	100	−3.8
NaCl	100	−3.2
$CaCl_2$	100	−2.1
Na_2HPO_4	100	−5.4
$MgCl_2$	100	−2.7
$FeCl_3$	100	−3.8
$CuNO_3$	100	−4.0
$ZnCl_2$	100	−4.6

existing in human urine (Table 3). The concentrations of these interfering substances, although rather higher than that of protein, mostly caused less than 5% relative error in the determination. The results indicate that, under the chosen incubation conditions, the selected interfering biomolecules gave no significant interference with the protein determination.

3.7 Clincal urine sample analysis

The correlation between this proposed method (choosing HSA as the standard curve) for the detection of human urinary protein and a commercially available method (Pyrogallol red–molybdate complex method) employed in the central laboratory of the Chengdu 7th People's Hospital was investigated. Accordingly, 50 urine samples from patients (protein concentrations of 100–4000 mg L^{-1}) were analyzed with the two methods and the obtained results were shown in Fig. 11. The equation of the line in the correlation plot is $Y = 1.04X + 6.56$ ($R = 0.988$, $N = 50$), which indicated this proposed method showed good correlation with the hospital reference method. Therefore, the determination of protein by this method is reliable and practical.

Conclusions

In summary, we have developed a sensitive and simple method for the determination of human urinary protein based on QDs labeling and ICP-MS detection. The low detection limit and wide linear range obtained using the present method provides a new possibility for bioanalyte quantification. The method presented here is not only sensitive and simple, but also proved to be reliable and suitable for practical application. This strategy is worthy of further exploration for the detection of nucleic acids, immunoassays and clinical diagnostics, and also has potential for simultaneous determination of multiple low-abundance biomarkers of interest by the combination of separation techniques and the sensitive ICP-MS detection.

Acknowledgements

Authors acknowledge the financial support for this project from the National Natural Science Foundation of China [No. 20775051 and 21075084] and the Sichuan Youth Science & Technology Foundation (No. 2009-18-409). The authors also thank Dr Shanlin Wang of the Analytical & Testing Center of Sichuan University for her generous help in TEM analysis.

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