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Sensitive and selective capillary electrophoretic analysis of proteins by zirconia nanoparticle-enhanced copper (II)-catalyzed luminol–hydrogen peroxide chemiluminescence

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A B S T R A C T

We report herein a sensitive, selective, convenient CE determination of heme proteins in complex matrices by a sodium-dodecyl-sulfate-assisted, zirconia nanoparticle-enhanced copper (II)-catalyzed luminol–hydrogen peroxide chemiluminescence (CCLHPCL). Introducing a segment of sodium dodecyl sulfate to the capillary after sample injection not only rendered selective detection by quenching the luminescence signals from the non-heme proteins but also owning to the suppressed protein adsorption, led to significant improvement in separation efficiency and detection sensitivity. The signals were further improved by addition of ZrO_2 nanoparticles to the chemiluminescence solution. Compared with the conventional CCLHPCL, the detection limits ($S/N=3$) were improved by 10.2–22.0 folds, with 7.8×10^{-9}, 3.3×10^{-9} and 1.5×10^{-9} M for three model proteins, viz, myoglobin, hemoglobin and cytochrome *C*, respectively. Because the method did not require sophisticated pretreatment, it was convenient to analyze heme proteins in complex matrices, as demonstrated, hemoglobin in human blood and spiked human urine samples.

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1. Introduction

Highly sensitive and selective protein determination techniques are increasingly required in the fields of basic discovery research, disease diagnostics, drug screening, and biodefense applications. Among these techniques, capillary electrophoresis (CE) is an attractive alternative to the traditional two-dimensional electrophoresis technique in protein analysis because of its wide dynamic range of sensitivity, high efficiency, rapidity, high resolution and ease of operation [1]. But CE encounters a number of shortcomings [2], of which the poor concentration detection limit is the most widely concerned [3]. To address this problem, numerous efforts have been explored, including in-column [4–6] and off-column [7,8] concentration, long pathlength (for the optical absorbance protocols) [9] and high-sensitivity detection strategies. However, developments in detection strategies aiming at sensitivity improvement are generally thought to be the most straightforward solution, and therefore draws persistent interest of analysts [10].

Chemiluminescence (CL) detection developed quickly in the past decade because it has the following advantages: (a) simplicity in instrumental design and therefore the low cost [11,12], (b) elimination of light scattering or instable background signal found in photo-absorbing protocols [13], and (c) high sensitivity comparable to laser-induced fluorescence (LIF) [14]. With regard to the proteome research, luminol–H_2O_2 is a successful CL system [15–17] for heme protein detection. In the CL reaction, Heme protein and hydrogen peroxide molecule form complex, a stronger oxidant responsible for the enhanced CL signal [18–20]. It was also reported [21] that presence of Cu(II) in luminol–H_2O_2 system led to the formation of protein–Cu(II) complex that was more efficient than Cu(II) alone for triggering the luminol peroxide luminescence. The strategy was termed as copper (II)-catalyzed luminol–hydrogen peroxide chemiluminescence (CCLHPCL). When coupled with CE, CCLHPCL extended the list of detectable proteins to non-heme proteins [22], but offered detection sensitivity lower than the conventional luminol–H_2O_2 approaches [4].

Use of nanometer-sized particles (nanoparticles, NPs) to enhance chemiluminescence has received considerable attention [23]. Studies suggest that the luminol–H_2O_2 reaction could be catalyzed on the surface of gold nanoparticles (GNPs) owning to the improved radical generation and electron-transfer processes [24], and/or to the stabilization of and consequently the increased fluorescence lifetime of excited luminol molecules upon adsorption [25]. Zirconia nanoparticles (ZrO_2 nanoparticles, ZNPs) have been widely used as catalytic materials. Their properties in CE as coating material [26–29] or stationary phases [30] have been investigated. Recently, they were employed for cataluminescence detection of gaseous species [31] and for electrogenerated-CL

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detection of tripropylamine [32]. So far, however, applications of ZNPs in chemiluminescence detection of biomolecules are less actively studied.

We herein explore the feasibility of improving the CE-CCLHPCL sensitivity and selectivity of proteins by using ZNPs and sodium dodecyl sulfate (SDS). SDS can form complex with proteins and suppress their adsorption to the capillary wall [1], benefiting high sensitivity. Moreover, introducing SDS might affect the interactions between Cu(II) and protein, opening a chance for tuning the selectivity for CL detection. In this report, parameters influencing CE separation efficiency, CL detection selectivity and sensitivity were studied. Finally, the method was applied to fortified human blood serum and human urine, and human blood samples.

2. Experimental

2.1. Chemicals, reagents and solutions

Myoglobin, albumin egg, γ-globulin (bovine), cytochrome *C* (horse heart), hemoglobin (bovine) and luminol (3-aminophthalhydrazide) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (fraction five) was from Amerso (Solon, OH, USA). Human serum was purchased from Chengwen Immuno (Beijing, China). Poly(vinylpyrrolidone) (PVP, MW 1 300 000) was the product of Alfa Aesar (Ward Hill, MA, USA). Tris(hydroxymethyl)aminomethane (Tris), acetic acid, hydrochloric acid, sodium dihydrogen phosphate, sodium hydroxide, copper (II) sulfate, hydrogen peroxide (30%), potassium sodium tartrate and SDS were bought from Beijing Chemical Plant (Beijing, China). Sodium tetraborate was from Tianjin Kemiou Chemical Reagent Development Center (Tianjin, China). ZNPs of 40 nm in average diameter (purity, 99.8%) were the product of Nanjing High-Tech (Jiangsu, China). Deionized water (Millipore, Bedford, MA, USA) was used for preparing solutions throughout the experiment.

The stock solution of luminol at 0.001 M was prepared with 0.1 M NaOH and stored in the dark. Stock solutions of $Na_2B_4O_7$, NaH_2PO_4 and SDS, at 100 mM each, and individual stock solutions of copper (II) sulfate and potassium sodium tartrate, at 1 mM each, were prepared with deionized water. The stock dispersion of ZNPs at 1000 mg L^{-1} was prepared in 30 mM tetraborate/phosphate buffer. All working solutions were prepared by appropriate dilution of the stock solutions with deionized water, and percolated (except for the buffers containing ZNPs) through 0.22 μm filters (Jiu Ding High Tech., Beijing, China) before use.

Ten milliliter human urine sample from a healthy volunteer was stored for 2 h at 4 °C after collection. After centrifugation for 10 min at 2000 rpm, the supernatant was percolated through a 0.22 μm filter. The pretreated urine samples, unspiked or spiked with human hemoglobin (kindly provided by the Beijing Blood Center) to 100 μg L^{-1}, were 4-fold diluted with deionized water before analysis.

The human blood sample (containing citrate, phosphate and dextrose as anticoagulant, stored at −20 °C) was the generous gift of Beijing Blood Centre (Beijing, China). Before analysis, it was defrosted and diluted with deionized water or 10-fold diluted human serum (human serum diluted with D.I. water, denoted as 1/10 human serum).

2.2. Apparatus

The CE–CL system consisted of a high-voltage power supply (Sanchuan High-Tech., Tianjin, China) and a laboratory-made CL detector. An on-column coaxial flow interface connecting CE and CL was manufactured following the work previously reported [33]. Briefly, the polyimide coating of 1 cm in length at one end of the separation capillary (50 cm × 75 μm, Yongnian Optical Fiber, Hebei, China) was removed. The bare end was inserted into a 530 μm i.d. reaction capillary (Yongnian Optical Fiber) to a depth of 1.6 cm, where a 0.5 cm detection window (made by removing the polyimide coating of the reaction capillary) centered. A four-way Plexiglas joint was employed to hold the separation capillary, the reaction capillary and the two CL solution-delivering capillaries in place (see Fig. 1 for the schematic diagram of the system). The chemiluminescence was monitored by a photomultiplier tube (PMT, R928, Hamamatsu Photonics, Shizuoka, Japan); the signal was collected by a compatible PC via a data acquisition unit (CT22, Qianpu, Jiangsu, China). For CE–UV experiments, separations were carried out in a 50 cm (with effective length of 40 cm) × 75 μm capillary; the solutes were monitored at 214 nm using a UV-10 detector (Johnsson, Liaoning, China).

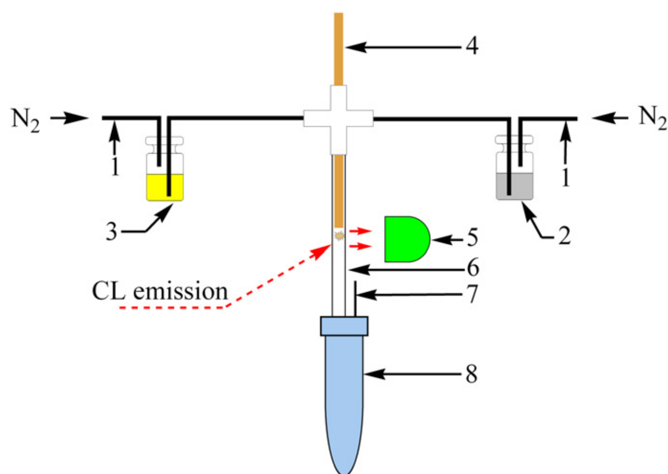

Fig. 1. Schematic diagram of CL system: (1) nitrogen gas inlet; (2) CL solution A; (3) CL solution B; (4) separation capillary; (5) PMT; (6) CL reaction capillary; (7) grounding electrode; (8) waste vial.

2.3. Capillary electrophoresis

The fresh separation capillary was flushed for 1 h with 0.5 M NaOH, followed by 0.5 h with deionized water, and finally for 0.5 h with buffer (unless otherwise stated, the buffer consisting of 20 mM sodium tetraborate and 10 mM sodium dihydrogen phosphate was employed). Before CE, the sample solution was introduced into the capillary by gravity for 20 s at a height of 35 cm, followed by a plug of SDS solution at certain concentration (5, 10, 20, 30 or 40 mM, the optimum was 20 mM). The length of the SDS plug was estimated based on the injection height and duration with a free software (CE Expert) provided by Beckman (https://www.beckmancoulter.com, last accessed on November 30, 2011). Separations were carried out at +14 kV under ambient temperature. Between two consecutive separations, the capillary was flushed for 2 min with buffer.

The theoretical plate number (N) of a peak was calculated using the formula $N=5.54\ (t/W_{0.5})^2$, where t and $W_{0.5}$ are the migration time and the peak width at the half height, respectively.

2.4. CL conditions

Two CL solutions prepared in run buffer, A and B, were employed for CL reaction. They were stored separately in two bottles (Fig. 1) and were pressure-driven to the reaction capillary at desired flow rates tuned with regulators. Unless stated otherwise, the following CL parameters were employed: CL solution A, 5×10^{-6} M copper (II) sulfate, 5×10^{-6} M luminol and 2.5×10^{-5} M potassium sodium

tartrate; CL solution B, 0.2 M hydrogen peroxide; the flow rates of CL solutions A and B were 4.2 and 10.5 μL min^{-1}, respectively.

3. Results and discussion

3.1. Influence of CE conditions

3.1.1. Polymer, surfactant and sample matrices

Adding polymers into buffer favors high efficiency and sensitivity because they can suppress the protein adsorption and can act as sieving matrix [1]. We attempted to separate proteins in a homogeneous medium containing 0.75% PVP, but no peaks could be detected in 30 min, likely due to the highly suppressed EOF (figures not shown). We found that six proteins could be detected (Trace a of Fig. 2A) by adding PVP to the buffer in anodic vial to 0.75% (m/v) and filling the separation capillary with polymer-free buffer. The proteins were separated in two peaks, with myoglobin, hemoglobin, and cytochrome *C* comigrated in the first peak, and bovine serum albumin, albumin egg and γ-globulin overlapped in the second one. The results agreed with the report [22] that the non-heme proteins could be detected using Cu(II) as catalyst. However, the detection sensitivity was low; additionally, the broad peaks, originating from the slow protein–wall adsorption/desorption kinetics, indicated poor separation efficiency.

Different concentrations of SDS, from 5 to 40 mM, were injected into the capillary, before or after sample loading. Injection of 12.5 mm × 20 mM SDS plug after sampling resulted in strong and sharp peaks (Trace b of Fig. 2A), enhancing CL signals by 11.4 and 4.3 folds, respectively, for myoglobin and cytochrome *C*. Changes in migration time reveal that the negative charge density of the proteins increased upon interaction with SDS. Such interaction also improved the resolution between the originally completely merged peaks. More importantly, the non-heme proteins, bovine serum albumin, albumin egg and γ-globulin, were not detected likely because their interactions with Cu(II) were inhibited. The protein–SDS interaction rendered selective detection of proteins, a promising feature for proteome analysis.

The selectivity of the CL detection was evaluated by comparison with CE–UV using human serum as a model background matrix. CE–CL depicts a nearly straight baseline on 1/4 human serum (Trace a of Fig. 2B); in contrast, strong peaks were detected on 1/10 human serum with CE–UV (Trace b of Fig. 2B). To test the method for the feasibility of detecting heme proteins in complex matrices, myoglobin, hemoglobin and cytochrome *C* were spiked in 1/10 human serum. The three proteins were successfully detected. Interestingly, the resolution between hemoglobin and cytochrome *C* improved such that they were baseline separated (Trace c of Fig. 2A), suggesting that the sample matrices affected the migration of the proteins. Besides human serum, we also studied bovine serum as sample matrix by spiking the three proteins in 1/10 and 1/4 bovine serums, the results were similar (figures not shown).

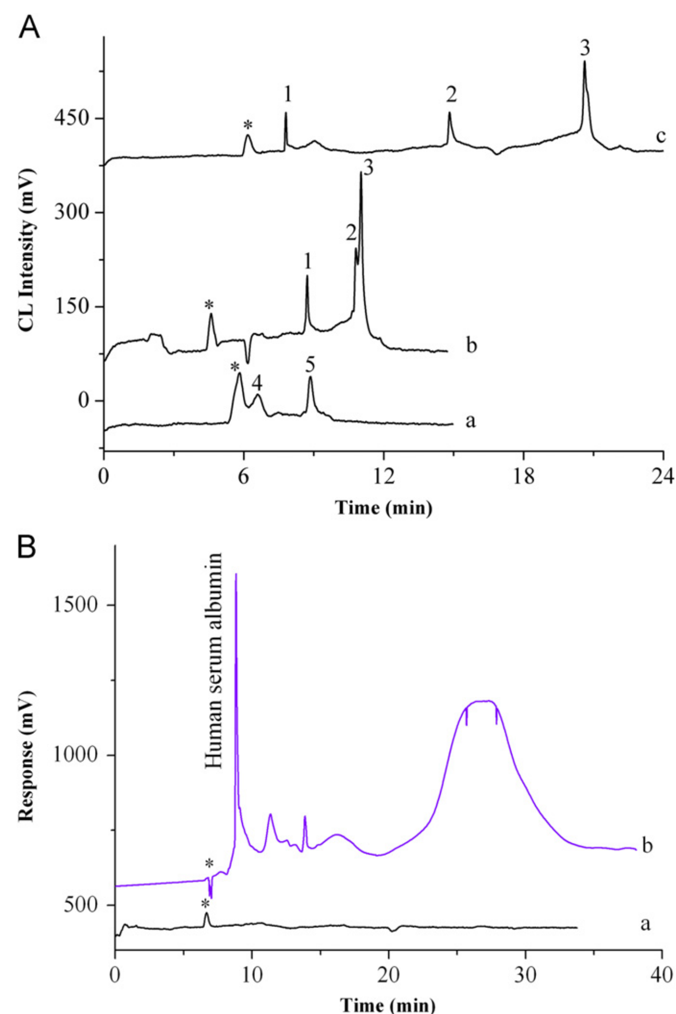

Fig. 2. Electropherograms of CE–CL (A) and CE–UV (B). The analytes in (A): a, described in the text; b, 100 mg L^{-1} myoglobin, 100 mg L^{-1} hemoglobin and 50 mg L^{-1} cytochrome C; c, proteins in b spiked in 1/10 human serum. The experimental conditions of trace a of (A) were described in the text; for the other traces in the figure, a 12.5 mm segment of 20 mM SDS was hydrodynamically injected after sample loading. Peak identities: *, electroosmotic flow (EOF); (1) myoglobin, (2) hemoglobin; (3) cytochrome *C*; (4) mixture of myoglobin, hemoglobin, and cytochrome *C*; (5) mixture of bovine serum albumin, albumin egg and γ-globulin. Traces were offset for clarity.

3.1.2. Buffer pH

Because the luminol–H_2O_2 chemiluminescence reaction requires alkaline environment [34,35], basic solutions of tetraborate/phosphate were employed as BGEs and for preparation of CL solutions. Influence of buffer pH was assessed by fixing the buffer concentration at 30 mM and altering tetraborate/phosphate ratio, from 1:2 to 6:1, corresponding to pH 7.9–9.4. The protein peaks were very low at pH 7.9 (Trace a of Fig. 3), probably owning

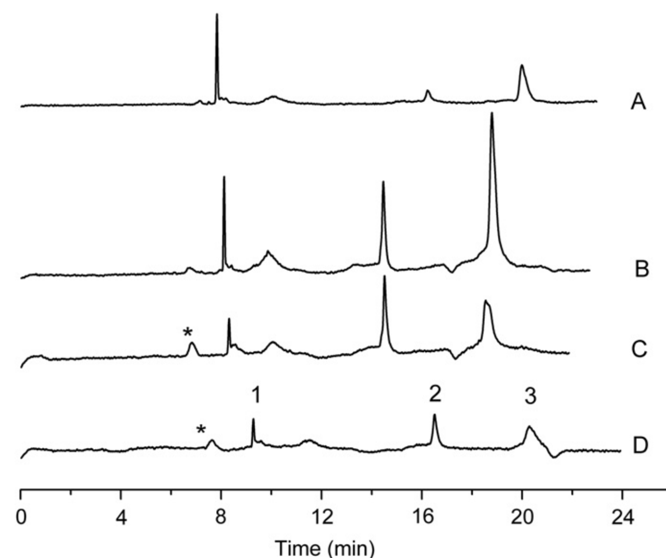

Fig. 3. Influence of buffer pH: (A) 1:2 (pH 7.9); (B) 2:1 (pH 9.1); (C) 4:1 (pH 9.3); (D) 6:1 (pH 9.4). Concentrations of the protein standards were 200, 200 and 50 mg L^{-1} for myoglobin, hemoglobin and cytochrome *C*, respectively.

to the low CL quantum yield at the relative low pH. The intensity of the peaks reached maximum at pH 9.1 (Trace b, tetraborate/phosphate ratio of 2:1), but progressively reduced with increasing alkalinity (pH 9.3, trace c, for ratio 4:1 and pH 9.4, trace d, for 6:1).

Besides affecting the luminol–H_2O_2 CL reaction, buffer pH imposes additional impacts on the luminescence intensity by altering the concentration of Cu(II), the catalyst. In our system, the concentration of Cu(II) was influenced by concentrations of three species that were pH-dependant: PO_4^{3-} through precipitation of $Cu_3(PO_4)_2$, tartrate through formation of Cu(II)–tartrate complex, and hydroxide ion through formation and polymerization of Cu(II) hydroxide [22,36]. Concentrations of PO_4^{3-} and tartrate virtually unchanged as the buffer pH varied from 9.1 to 9.4 (see Table 1S and 2S of the supporting materials). Changes in OH^- concentration accounted for the reduced chemiluminescence because it approximately doubled from pH 9.1 to pH 9.4 (Table 1S), facilitating the formation of $Cu(OH)_2$ (K_{sp}=2.2×10^{-20} [37]) precipitations. So, in CCLHPCL high buffer alkalinity hinders the luminescence by reducing the concentration of free Cu(II).

3.1.3. Buffer concentration

High buffer concentration minimizes the wall-adsorption of proteins, leading to high CL responses and high separation efficiency. However, the Joule heating arising from strong ionic strength will generate air bubbles during CE, which is the main source for elevated baseline noise and degraded reproducibility in migration times and peak areas. Moreover, increased concentration of PO_4^{3-} in the buffer would suppress the CL emission according to the discussion in Section 3.1.2. Studies on BGEs of 18, 30, 42, 60 mM tetraborate/phosphate (at a fixed ratio of 2:1) suggested that buffer of 30 mM is optimal (Fig. 1S of the supporting information).

3.2. Influence of CL parameters

3.2.1. CL-reagent concentration

The concentrations of the CL reagents directly influence the CL intensity and baseline noise. It was found by experiments that luminol concentration of 5.0×10^{-6} M was optimal. Based on this, the influences of H_2O_2, copper (II) and tartrate were studied.

Hydrogen peroxide at 0.2 M rendered highest signal-to-noise ratio. Further increasing H_2O_2 concentration resulted in high baseline noise and occasional current breakdown during separation, which might be caused by the oxygen bubbles produced from H_2O_2 decomposition. Experiments on CE–CL with Cu(II) of 1.0×10^{-6} M to 2.5×10^{-5} M revealed that 2.5×10^{-6} M was the favorable concentration, at which the detection sensitivity of the proteins approximately doubled (Fig. 4). The results also suggest that copper (II) is an effective catalyst in the presence of SDS and ZNPs. Copper (II) catalyzes luminol–H_2O_2 redox reaction by forming copper (II)–protein complex via histidine groups of the protein molecules [22,38]. Its catalytic activity reaches maximal when two of its four coordination sites are occupied by ligands [22,36]. In order to satisfy such requirement, potassium sodium tartrate was added into the CL solution as a masking reagent to facilitate the formation of Cu(II)–protein complex by preventing polymerization of Cu(II) hydroxide from hydrolysis reaction in alkaline solution. Variation in peak heights of protein as a function of the tartrate concentration between 5×10^{-6} and 1.25×10^{-4} M depicted a maximum at 2.5×10^{-5} M (figures are not shown). The decreased CL intensity in the presence of high-concentration tartrate might be the consequence of the suppressed Cu(II)–protein coordination.

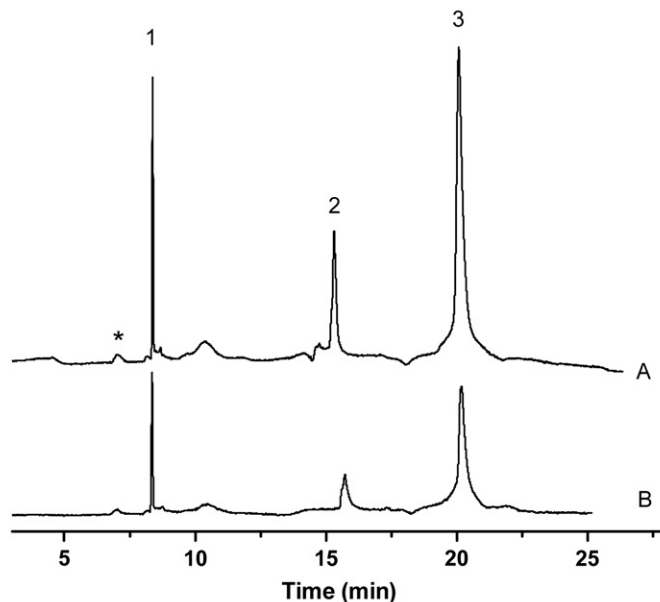

Fig. 4. Influence of Cu(II) on the detection sensitivity. The buffer: 30 mM tetraborate/phosphate at pH 9.1. The CL conditions: (A) 2.5×10^{-6} M copper (II), 2.5×10^{-5} M luminol and 2.5×10^{-5} M potassium sodium tartrate; (B) the CL solution A devoid of copper (II).

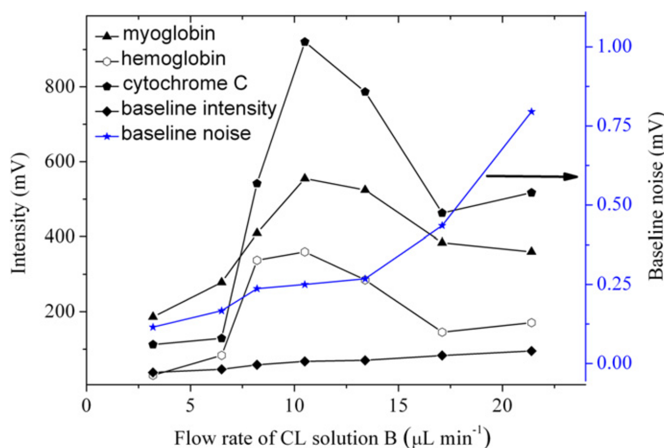

Fig. 5. Variation of CL signal as a function of flow rate of CL solution B. During experiments, the flow rates of CL solutions A and B were kept at a constant ratio of 2:5; the bulk flow rate of the whole CL solution was the sum of A and B, which could be obtained by multiplying the corresponding values in the figure by a factor of 1.4.

3.2.2. Flow rates of the CL solutions

The strongest CL response was obtained at flow rates of 4.2 and 10.5 μL min^{-1} for CL solutions A and B, respectively (Fig. 5). High-rate flow delivers more CL reagents to the reaction capillary and consequently promotes the luminescence reaction. But it boosts the undesired background Cu^{2+}–luminol–H_2O_2 reaction that is responsible for the high baseline intensity, dilutes the sample and therefore reduces the CL signal of proteins, and is ease to generate flow turbulence at the CE/CL interface and brings about enhanced baseline noise.

3.2.3. ZNPs

ZNPs were added to the CL solution in the range 5–100 mg L^{-1} to investigate their influences on detection. The peak intensity of proteins enhanced with increasing amount of ZNPs. Highest CL signals were achieved in the presence of 50 mg L^{-1} ZNPs, which provided detection sensitivity improvements of 2.3, 2.7, and

2.9 folds for myoglobin, hemoglobin and cytochrome *C*, respectively. Nanoparticles have on their surfaces multiple active centers that favor H_2O_2 adsorbing and consequently decomposing and yielding free radicals that can accelerate luminol oxidation [25]. Furthermore, NPs can immobilize the produced luminophore, the excited 3-aminophthalate ion [39], inhibiting their collisions with other molecules/ions in the solution and thereby alleviating the collision-induced fluorescence quenching.

According to the results, combination of ZNP and SDS-plug (described in Section 3.1.1) strategies enhanced the detection sensitivity by 10.2–22.0 folds relative to the original CCLHPCL approach.

3.3. Performance and application

The limits of detection (LODs, $S/N=3$) of myoglobin, hemoglobin and cytochrome *C* in 1/10 human serum were 7.8×10^{-9}, 3.3×10^{-9} and 1.5×10^{-9} M, respectively, lower than the methods employing conventional luminol–H_2O_2 CL detection [4] or CCLHPCL detection [22] that were obtained from standards in water matrices. The theoretical plate numbers for the three proteins were between 2.6×10^4 and 1.2×10^5 (in 50 cm separation capillary), higher than the existing results on CE–CL analysis of proteins [10,22]. We propose that injection of SDS plug in this experiment successfully suppresses the wall-adsorption of the protein, leading to the observed high separation efficiency; moreover, addition of ZNPs to the CL solution catalyzes the CL reaction, thereby minimizes peak broadening.

To further demonstrate its applicability, the method was applied to analyze urine (unspiked and spiked with hemoglobin) and blood samples from health people. The samples were not sophisticatedly treated except for dilution with D.I. water or with 1/10 human serum (for blood sample only). Although the urine matrix components generated signal in the CE–CL conditions, they did not interfere the detection of the spiked hemoglobin (traces a and b of Fig. 6), whose LOD was estimated to be 2.0×10^{-9} M, slightly better than that prepared in the matrix of 1/10 human serum. Electropherograms from blood samples prepared in different matrices are similar (traces c and d), suggesting the robustness of the developed CE–CL method. The concentration of hemoglobin was determined by standard addition to be 0.15 g mL^{-1}, lying within the normal reference ranges (13.5–18 g dL^{-1} for adult male and 12–16 g dL^{-1} for adult female. From http://www.cancerquest.org/complete-blood-count-rbc.html, last accessed on November 30, 2011). The results indicate that the method allows conveniently analyzing heme proteins in complex sample matrices.

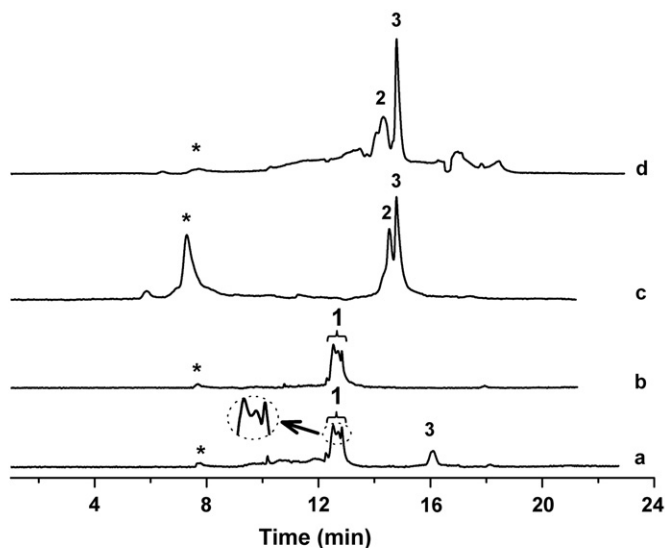

Fig. 6. Electropherograms of human urine and human blood sample: (a) 4-fold diluted human urine sample that was originally spiked with 100 mg L^{-1} hemoglobin (human); (b) 4-fold diluted human urine sample; (c) human blood sample 500-fold diluted with 1/10 human blood serum; (d) human blood sample 1000-fold diluted with deionized water. Concentration of ZNPs was 50 mg L^{-1}. Peak identities: *, EOF; (1) unknown components in human urine; (2) unknown components in human blood; (3) hemoglobin.

4. Conclusions

We developed a method for detecting heme proteins in the presence of complex matrices such as human blood serum, human urine or human blood samples based on the SDS-assisted, ZNP-enhanced CCLHPCL emission. Introducing SDS segment after sample injection not only improved detection sensitivity because the wall adsorption of proteins was effectively suppressed, but also provided selectivity for heme protein detection because the CL emission of non-heme proteins was suppressed by formation of the SDS–protein complex. Peak heights of the proteins were further improved by addition of ZNPs to the CL solution. Combination of the above approaches dramatically improves CCLHPCL detection sensitivity and selectivity. The method developed is robust and is capable of convenient, selective and sensitive detection of proteins in complex matrices without sophisticated pretreatment techniques.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2012.04.016.

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