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Research Article

Development of sensitive metalloporphyrin probes for chemiluminescent imaging detection of serum proteins

The development of metalloporphyrin- (ferric protoporphyrin IX chloride (FePP), cobalt (III) protoporphyrin IX chloride, copper (II) protoporphyrin IX) enhanced chemiluminescent (CL) imaging detection of serum proteins after PAGE is described in this article. The detection is based on the catalytic activity of metalloporphyrins, especially FePP, in the CL reaction of the luminol–H_2O_2 system. Some relatively low abundant proteins such as hemopexin (Hpx) and complement C4 are sensitively detected by FePP-enhanced CL imaging. Other proteins such as haptoglobin, apolipoprotein A-1, complement C3, and α-1-antitrypsin are also detected and identified by MS and MS/MS techniques. Detection limit of Hpx is as low as 20 ng, without the need of expensive antibodies or tedious immunoassay procedures. The mechanism of the proposed method is discussed employing standard proteins. The application to the analysis of different protein patterns in healthy people and in Thalassemia patients is being investigated.

Keywords:
Chemiluminescent imaging / Hemopexin / Human serum proteins / Metalloporphyrin / PAGE
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1 Introduction

Serum is one of the most commonly tested body fluids for clinical diagnosis. It contains a high concentration of various proteins that are involved in numerous physiological processes including metabolism, transport, hemostasis, and immune responses [1]. Serum proteins may often serve as indicators for the diagnosis of diseases. One of the driving forces for proteome analysis is the discovery of biomarkers, proteins that change in concentration or state in associations with a specific biological process or disease. Therefore, the study of serum proteome is a powerful diagnostic tool to define the onset, progression, and prognosis of human diseases [2–4].

1-DE and 2-DE are common methods used for separating proteins from animals and microorganisms. After separation, various detection methods can be used: CBB-R250 staining is a traditional and commonly used method in the laboratory; silver staining is a method with multistep procedures, which is considered to be much more sensitive than CBB staining [5, 6]; and immunoassays are alternative methods to detect low abundant proteins because of their specificity for the target analyte and their high sensitivity [7, 8]. Chemiluminescence (CL)-based detection was recently introduced in the area of biochemical research. It is based on the sensitive CL reaction of luminol and H_2O_2 in the presence of a catalyst. Our group has developed a direct CL imaging method to detect serum proteins in polyacrylamide gels [9]. This technique has greatly simplified the procedures for protein detection compared with CBB-R250 staining and provides high sensitivity and selectivity. Specific proteins that can catalyze the CL reaction of luminol and H_2O_2 can be easily detected. Unfortunately, most proteins cannot generate CL emission, and this disadvantage confined the method to clinical applications to some extent. Hence, much work has been done to develop a more sensitive and comprehensive CL imaging detection method [10–13].

Many metalloporphyrins have been used as mimetic enzyme of horseradish peroxidase in fluorescence and CL analysis. The catalytic effect of metalloporphyrins for the luminol CL system has led to extensive research [14–17]. Literature data also make clear that metalloporphyrins can bind to proteins [18–20]. Ikarlyama and Suzukl developed a

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Abbreviations: CoPP, cobalt (III) protoporphyrin IX chloride; **CuPP,** copper (II) protoporphyrin IX; **CL,** chemiluminescent; **FePP,** ferric protoporphyrin IX chloride; **Hpx,** hemopexin

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method termed luminescence catalyst immunoassay in which HSA is used, labeled by ferric protoporphyrin IX chloride (FePP) to catalyze the CL reaction of luminol and H_2O_2 [14]. FePP binds tightly, sometimes covalently, through protein sequences that commonly contain a histidine–methionine pair or bis-histidine. It is generally known that hemopexin (Hpx) and HSA bind FePP with high and medium affinity, respectively [20, 21]. As a structural analogue to FePP, other metalloporphyrins may also exhibit such affinities toward proteins. Since metalloporphyrins can not only bind to proteins but also show catalytic activity in the luminol and H_2O_2 CL reaction, they may be promising probes for the CL imaging method for detecting proteins.

The purpose of establishing a simple and sensitive method for the detection of serum proteins is performed employing metalloporphyrin-enhanced CL imaging detection combined with PAGE. The effect of three metalloporphyrins (FePP, cobalt (III) protoporphyrin IX chloride (CoPP), and copper (II) protoporphyrin IX (CuPP)) on the CL imaging detection is investigated. Various proteins including some relatively low abundant proteins that cannot be stained by CBB-R250 are sensitively detected by the proposed method. This is based on the fact that metalloporphyrins can bind to proteins and catalyze the luminol–H_2O_2 CL reaction. The presently proposed method is applied to the analysis of different protein patterns comparing healthy people and Thalassemia patients. The system offers simple and inexpensive procedures. Furthermore, it does not produce radioactive pollution nor noxious materials.

2 Materials and methods

2.1 Reagents

All reagents were of AR grade. Luminol (3-aminophthalic hydrazide) was obtained from Acros Organics (New Jersey, USA). HSA and IgG were purchased from Sigma (St. Louis, MO, USA). Molecular-weight markers for proteins were purchased from Amersham Pharmacia Biotech (USA). Ampholytes (pH 4–6) were from Serva (Heidelberg/New York). FePP was obtained from Fluka (Switzerland). CoPP and CuPP were obtained from Frontier Scientific (Carnforth/Lancashire, UK). FePP, CoPP, and CuPP were dissolved in 0.1 M NaOH. The stock solution of luminol (0.1 M) was prepared by dissolving luminol in 0.1 M NaOH solution without purification; it was further diluted with 0.1 M NaOH to the requested concentration. MilliQ water (Millipore, Bedford, MA) was used to prepare solutions.

Blood samples of healthy subjects were obtained from the affiliated hospital of the Beijing Normal University. After careful settlement for 2 h, the supernatant was extracted as serum with a minisample collector, then centrifuged at 2500 rpm for three times (each time 10 min). Serum samples of β-Thalassemia patients were obtained from Guangzhou Children's Hospital (Guangzhou, China). Sera were stored at -20°C for 60 days.

2.2 Gel electrophoresis

Nondenaturing 1-DE and 2-DE were carried out as previously described [12]. Briefly, the 1-DE was performed at a separation temperature of 20°C in a vertical discontinuous gel system, consisting of separating (7.5%, *m*/*v*) and stacking (4.0%, *m*/*v*) gels (82 mm × 82 mm × 1.5 mm). The voltage was set at 130 V, then turned down to 100 V when the sample entered the separating gel. For 2-DE, proteins were subjected to IEF in the absence of denaturants employing column gels (2 mm id × 75 mm) at 20°C. IEF was run at 200 V for 30 min and continued at 400 V constant voltage for 15 h. After that, gels were transferred onto the second-dimension-slab gels (7.5%, *m*/*v*; 82 mm × 75 mm × 1.5 mm). The voltage was set at 120 V.

2.3 Detection of proteins

2.3.1 Direct CL imaging detection

After removal from the electrophoresis mold, the polyacrylamide gel was washed with deionized water to avoid the interference of rudimental electrophoresis buffer. Then it was spread on an even glass in a dark room. Hydrogen peroxide solution (0.5% v/v) was sprayed onto the gel for 20 s, and then luminol (2.0×10^{-4} M, pH∼13) for 1 min. Next the gel was quickly drained with filter paper, and covered with a sheet of transparency film. Immediately after, an X-ray film was exposed to the gel for 1 min, then developed for 1 min, and submerged into the fixing agent for 2 min. The films were scanned with a scanner and the results were transferred to the computer.

2.3.2 CBB-R250 staining detection

After separating, the gel was stained in the CBB-R250 (0.1 g/100 mL) solution (35% methanol–10% acetic acid in distilled water) for 2 h, next it was washed and submerged into the ethanol–acetic acid solution (10%/7% in distilled water) to destain for about 10 h.

2.3.3 FePP-enhanced CL imaging detection

When the gel was taken out of the electrophoresis mold, it was washed with deionized water and then submerged into the solution of FePP for 15 min. To eliminate the superfluous FePP, deionized water was used for washing the gels. The following procedures were accomplished in a dark room as described by the direct CL imaging detection method. The results were transferred to the computer.

3 Results and discussion

3.1 Investigation of the mechanism of CL enhancement by metalloporphyrins

The experiment was designed to develop new probes for the detection of serum proteins that cannot be detected by the direct CL imaging. According to the literature reports, FePP is a metalloporphyrin that possesses both abilities of binding to proteins and catalyzing the luminol CL reaction [14, 20, 22]. Therefore, FePP was first employed to investigate the enhancement effect to CL imaging detection.

3.1.1 Study of FePP binding to HSA

HSA is known to bind FePP with medium affinity [20]. In order to determine whether it can be detected when FePP is used as a probe, an experiment was carried out as follows: (i) different amount of HSA were first mixed with FePP solution for the interaction of the two reagents, (ii) all the samples ran 1-DE to avoid the interference of free FePP, and (iii) the gel was detected by CL imaging method. As illustrated in Fig. 1, the CL signals increased along with the increase in HSA concentrations from lane 1 to lane 7; no CL was generated without FePP in lane 8. Because free FePP had run out of the gel, it could be easily deduced that the only species responsible for the CL emission is the FePP–HSA complex. Hence, FePP can be used as a CL probe in protein detection.

3.1.2 Detection of different proteins

To investigate the CL enhancement of FePP on different proteins, IgG and HSA were compared on nitrocellulose membranes. HSA (2 mg/mL) and IgG (5 mg/mL) were spotted onto two NC membranes. One of them was submerged into the solution of FePP. Next, hydrogen peroxide and luminol were spread on it. On the other membrane, hydrogen peroxide and luminol were directly spread without labeling FePP. The comparison of both membranes is shown in Fig. 2. In Fig. 2B, no CL signal appears indicating that both IgG and HSA cannot catalyze the luminol CL reaction. After labeling FePP, both proteins can be easily detected (Fig. 2A). In addition, the spot of HSA generated much stronger CL than the spot of IgG, though it had a lower concentration. The results indicate that the catalyst FePP binds proteins with different affinities, which leads to the different detection sensitivities of proteins.

Further investigation was performed employing protein markers that contain the following proteins: thyroglobulin, ferritin, catalase, lactate dehydrogenase, and albumin. After separation by PAGE, one gel was detected by direct CL imaging. As illustrated in Fig. 3A, only ferritin and catalase can produce CL signals. Another gel was first immersed in the solution of FePP before detection. As a result, all the proteins can be easily detected by the proposed method. Again, the CL intensities of protein bands are different.

As demonstrated earlier, different proteins can be detected by FePP-enhanced CL imaging, which is achieved by the binding of FePP to proteins. Through capturing the CL emission of FePP, the correlative proteins can be detected. The high FePP affinity of proteins such as Hpx is mainly generated by the coordination of Fe(III) with the two histidine residues in the proteins. Other proteins that do not have such biological function would bind FePP by other forces such as π–π interaction [21]. This difference in binding ability determines the CL detection sensitivities of

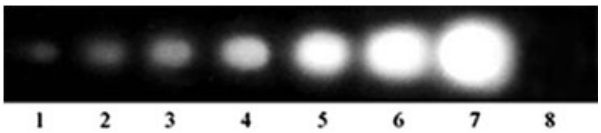

Figure 1. Study of FePP binding to standard HSA. 1–7 contains the same concentration of FePP (5×10^{-3} mg/mL). HSA concentration was gradually increased: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, and 0.15 mg/mL. (8) 0.15 mg/mL HSA without FePP.

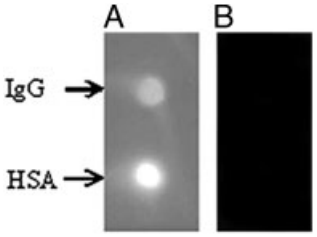

Figure 2. Enhancement of CL signal by FePP on nitrocellulose membrane. (A) Membrane with 15-min immersion into the solution of FePP. (B) Membrane without immersion into the solution of FePP. The loading volumes of HSA (2 mg/mL) and IgG (5 mg/mL) are both 2 μL.

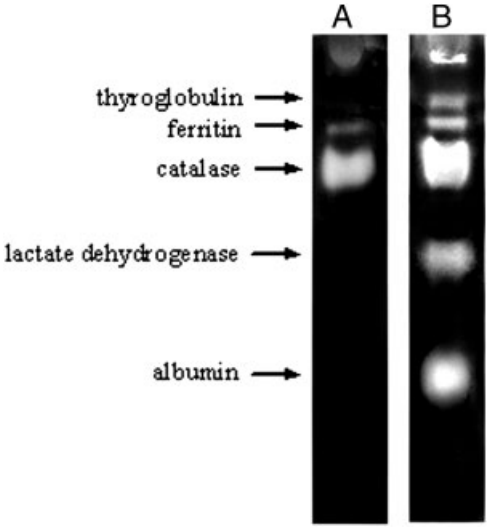

Figure 3. Comparison of direct CL imaging and FePP-enhanced CL imaging detection of protein markers. (A) Direct CL imaging. (B) FePP-enhanced CL imaging.

various proteins, just as exemplified by the comparison of different proteins in the former experiments. It is presumed that a protein can be more sensitively detected when it has a higher FePP affinity.

3.2 Enhancement of CL signal by metalloporphyrins

3.2.1 Preliminary results in 1-D gels

Preliminary experiments of detecting serum samples were performed with three metalloporphyrins: CuPP, CoPP, and FePP. To our knowledge, the effect of CuPP and CoPP on the luminol CL system has not been reported previously, and therefore was tested first. An experiment was carried out by directly adding both metalloporphyrins to the luminol–H_2O_2 mixing solution, respectively. It was observed that while CuPP produced very little CL emission when added to the luminol–H_2O_2 solution, CoPP generated an obvious signal. Further investigation was performed in polyacrylamide gels to detect proteins. Serum samples containing different metalloporphyrins were separated by 1-DE and then detected by CL imaging. The results were consistent with the previously tested CL results (Fig. 4). When CuPP was added to the serum sample, the result was the same as the blank one that did not contain any metalloporphyrins; only some weak bands of Hp were detected. When CoPP was added as a probe, CL signals of various proteins were easily captured. However, it was much less sensitive than FePP-enhanced CL imaging since the concentration of CoPP added to serum was over 100 times higher than that of FePP. On the basis of the obtained results, it could be concluded that FePP is the best reagent out of the three metalloporphyrins added to enhance the CL signals. Therefore, FePP was used for further experiments.

The Cu^{2+} is well known as an effective catalyst of the luminol–H_2O_2 CL reaction. However, when Cu^{2+} forms saturated complexes with some organic ligands, its catalytic activity disappears immediately [23, 24]. In this investigation, CuPP was the saturated complex of Cu^{2+} and porphyrin, and therefore cannot be used as a probe in the CL imaging detection.

3.2.2 Enhancement of CL signals by FePP in 2-D gels

Proteins bind FePP with different affinities, and not all FePP–protein complexes can undergo electrophoretic separation. Weak complexes may decompose under the conditions of electrophoresis. Thus, these proteins cannot be detected by CL imaging. In order to improve the detection ability, an experiment was performed by labeling FePP directly on gels that contained separated proteins, so that FePP–protein complexes would not go through the electrophoretic separation.

2-DE is a classical technique for the parallel display of many proteins from complex biological samples. It provides much more information on the protein composition in serum when compared with 1-DE. Therefore, 2-DE was employed to improve the detection ability. Two gels were run in parallel and one gel was detected by direct CL imaging without labeling FePP; the other was first submerged into the solution of FePP before detection. The comparison of the CL imaging results with and without FePP as a probe is illustrated in Fig. 5. From Fig. 5A, it can be seen that only a narrow range of proteins can be detected. However, many more proteins in human serum can generate CL emission when using FePP-labeled CL imaging. As the gel submerged into the solution containing FePP, the latter was bound to proteins. By capturing the CL emission of FePP, the spots of the proteins can be detected. Moreover, the detection sensitivity is also improved.

In comparison with other probe-enhanced CL imaging detection methods in previous work, FePP-enhanced CL imaging detection greatly improved in detection sensitivity as well as with respect to detection resolution. Furthermore, metalloporphyrins can be one-step labeled to proteins without the requirement of special instruments. [12, 13]

3.2.3 Protein identification

To determine the proteins detected by FePP-enhanced CL imaging, some protein spots indicated by the numbered

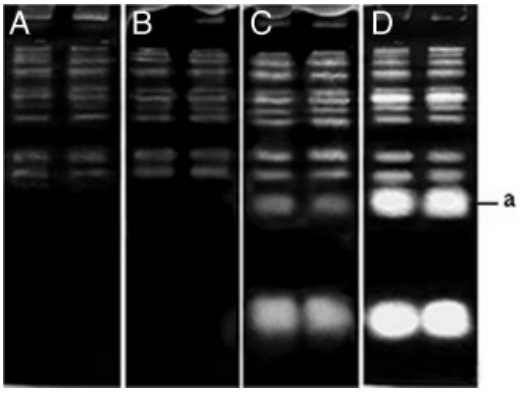

Figure 4. Effect of metalloporphyrins on CL imaging detection. (A) Blank serum. (B) Serum sample with CuPP (0.60 mg/mL). (C) Serum sample with CoPP (0.57 mg/mL). (D) Serum sample with FePP (5.2×10^{-3} mg/mL). Human sera diluted 1:10 in sample buffer; loading volume: 15 μL.

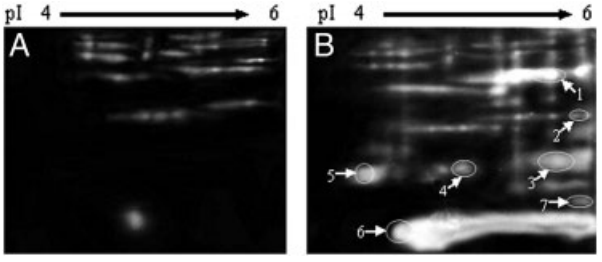

Figure 5. Comparison of direct CL imaging and FePP-enhanced CL imaging detection of serum proteins after 2-DE. (A) Direct CL imaging detection. (B) FePP-enhanced CL imaging. Human serum diluted 1:6 in sample buffer; loading volume: 50 μL.

arrows on the gel image in Fig. 5B and band "a" in Fig. 4 were identified by MS and MS/MS techniques. The research results are shown in Table 1 (see Supporting Information). Among these spots, different proteins are identified: spots 1, 2, 6, and 7 stand for Ig α-1 chain C region, apolipoprotein A-1, α-1-antitrypsin, and serum albumin, respectively. Spot 4 is a mixture of complement C3 and albumin. Moreover, two relatively low abundant proteins of complement C4 and Hpx are identified. Spot 5 stands for complement C4. Band "a" in 1-D gel is identified as Hpx, and the corresponding location in 2-D gel is spot 3 as it also stands for Hpx.

3.3 Optimization of the concentration of FePP

3.3.1 Effect of FePP concentration on CL signal in 1-D gels

A series of concentrations of FePP was tested to establish the proper concentration for protein detection. The concentrations of FePP added to sera ranged from 0.94 to 9.67 μg/mL. Samples were separated by 1-D PAGE and then detected by CL imaging. The results (Supporting Information Fig. 1) show that the concentrations of FePP strongly affect the results of CL emission. CL signals of protein bands were greatly enhanced along with the increase of FePP. When the concentration of FePP increased to high values, the CL emission of the proteins achieved high intensities and the background signal also increased and the resolution of protein bands decreased. Therefore, a proper concentration of 5.20 μg/mL was chosen for obtaining best CL imaging results.

3.3.2 Effect of FePP concentration on CL signal in 2-D gels

The effect of the concentration of FePP was also investigated in 2-D gels. Gels with separated serum proteins were submerged into solutions of FePP (1.3, 2.6, and 3.9 μg/mL) for 15 min. The results are illustrated in Supporting Information Fig. 2. It was observed that the CL signal increased with increasing FePP concentrations; therefore, higher concentrations were preferred. On the contrary, higher concentrations produced a higher background, leading to lower signal/noise ratios. Hence, 2.6 μg/mL was considered as the best concentration of FePP solution.

3.4 Comparison of FePP-enhanced CL imaging and CBB-R250 staining

3.4.1 Comparison of both methods in 2-D gels

CBB staining is the most commonly used gel staining method in the laboratory, favored for its good sensitivity and versatility. To obtain an idea about the detection ability, the proposed method was compared with CBB-R250 staining. Serum samples were first separated by 2-DE. After that, one gel was detected by FePP-enhanced CL imaging; the other was detected by CBB-R250 staining. The comparison of two results is shown in Fig. 6. It was observed that FePP-enhanced CL imaging can detect more protein spots. Some proteins can be easily detected by the proposed method, but can hardly be found in the stained map (the rectangle regions are shown in Figs. 6A and B). It made clear that FePP-enhanced CL imaging detection was more sensitive than the commonly used CBB-R250 staining method for most proteins.

3.4.2 Sensitivity evaluation

To obtain an idea about the detection sensitivity, experiments were carried out by using serially diluted serum samples containing FePP. Comparing FePP-enhanced CL imaging with CBB-R250 staining (Fig. 7), it was observed that FePP-enhanced CL imaging was slightly more sensitive than CBB-R250 staining for most proteins. However, for the detection of Hpx, FePP-enhanced CL imaging was much more sensitive. There were no visible Hpx bands in the CBB-R250 staining map, but in the CL imaging data, the Hpx could still be detected when serum was diluted 1/512. Taking an average Hpx concentration of 0.7 mg/mL in healthy adults' serum [25, 26], the detection limit of Hpx was as low as 20 ng. This low detection limit is contributed to the high affinity of FePP to Hpx and its high catalytic activity to luminol CL.

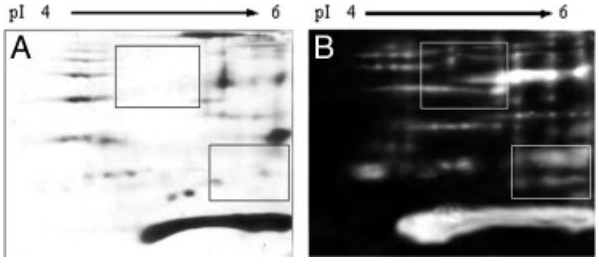

Figure 6. Comparison of CBB-R250 staining and FePP-enhanced CL imaging detection of serum proteins. (A) CBB-R250 staining detection. (B) FePP-enhanced CL imaging detection. Human serum diluted 1:6 in sample buffer; loading volume: 50 μL.

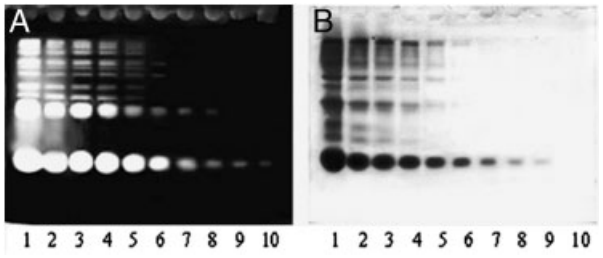

Figure 7. Comparison of the sensitivities of FePP-enhanced CL imaging and CBB-R250 staining. (A) FePP-enhanced CL imaging. (B) CBB-R250 staining. Dilution ratio of the serum samples: (1) 1/8, (2) 1/32, (3) 1/48, (4) 1/64, (5)1/108, (6) 1/256, (7) 1/512, (8) 1/1024, (9) 1/1536, and (10) 1/2048. Loading volume: 15 μL.

The intensities of protein bands in the X-ray film of Fig. 7A were analyzed by Gel Documentation and Analysis System (UVP-EC3). Taking an average HSA concentration of 40 mg/mL in healthy adults' serum, the calibration curve is shown in Fig. 8. The linear dynamic range of HSA in FePP-enhanced CL imaging was 0.2–20 μg ($R^2 = 0.983$).

3.5 Application to serum samples from healthy donors and Thalassemia patients

Hpx is a plasma glycoprotein mainly expressed in liver. The concentration of Hpx fluctuates under certain pathological conditions such as hemolytic anemia, porphyrias, chronic liver diseases. Determination of serum Hpx was used to assess the severity of hemolytic processes such as Thalassemia, sickle cell disease, as well as in patients with cardiac-valve protheses [7, 26].

Since FePP-enhanced CL imaging offers rapid and sensitive detection of serum proteins, sera from healthy people and Thalassemia patients were analyzed to investigate the feasibility of the proposed method. Sera from three β-Thalassemia intermedia patients and one β-Thalassemia major patient were tested to find the difference with healthy people. All samples from patients and healthy people groups were rigidly controlled under the same experimental conditions. Representative 1-DE maps from two individual samples are shown in Figs. 9 and 10. The CL imaging map of patient's serum differed from the healthy people in the Hp and the Hpx areas (Fig. 9A): it was easily observed that there was a less visible Hp band for the patient pattern and the Hpx value was markedly decreased. Figure 10A also illustrates that the protein pattern of the β-Thalassemia major patient was quite different when compared with normal serum: only two slight bands were detected in the Hp region and Hpx was almost undetectable. However, when detected by CBB-R250 staining, the differences between healthy people and β-Thalassemia patients were not quite clear. As shown in Figs. 9B and 10B, the detection of different Hp patterns was interfered by the high background, Hpx being undetectable by CBB-R250 staining.

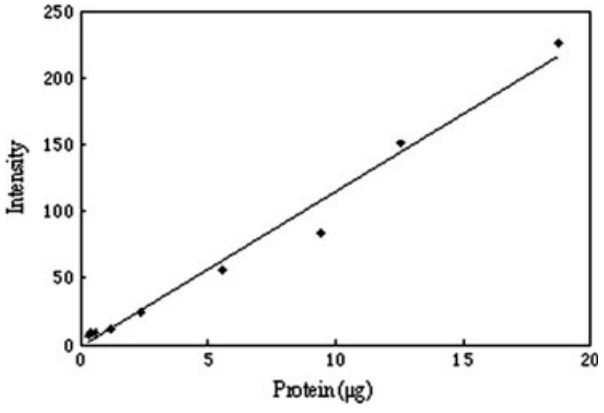

Figure 8. Calibration curve of HSA in FePP-enhanced CL imaging.

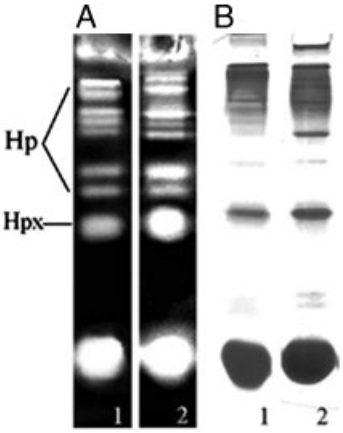

Figure 9. Detection of serum samples from β-Thalassemia intermedia patients and healthy people. (A) FePP-enhanced CL imaging. (B) CBB-R250 staining. (1, β-Thalassemia intermedia patients; 2, healthy people).

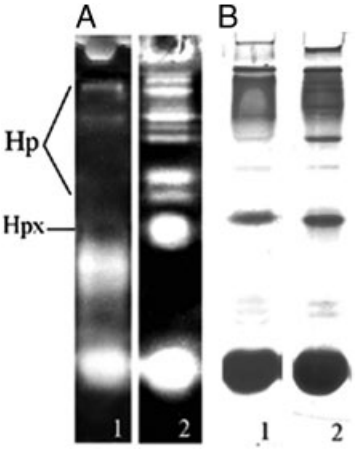

Figure 10. Detection of serum samples from β-Thalassemia major patients and healthy people. (A) FePP-enhanced CL imaging. (B) CBB-R250 staining. (1, β-Thalassemia major patients; 2, healthy people).

The results were consistent with earlier observations in literature [27–29]. Using the present method, we detected serum Hpx decrease in Thalassemia patients, particularly observed in Thalassemia major. It appeared that the determination of Hpx had potential applications in the assessment of the severity of Thalassemia. The role of Hpx as a diagnostic marker for other pathological conditions will be undertaken in a further study.

4 Concluding remarks

The present study demonstrates that metalloporphyrins-enhanced CL imaging detection combined with PAGE represents an effective means to detect serum proteins.

High sensitivity is achieved when FePP is used as a probe. With the proposed method, various serum proteins including some relatively low abundant proteins can be easily detected. The successful detection of proteins is due to the binding of FePP to proteins and the CL character of the former. The application to the detection of patients' sera represents the advantages of FePP-enhanced CL imaging over classical CBB-R250 staining. As a novel CL detection method, it shows significant biological analytical potentials in biochemistry and in future molecular biology.

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The authors have declared no conflict of interest.

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