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Fast haptoglobin phenotyping based on microchip electrophoresis

Bingrong Huang[a], Changgang Huang[a,b], Pingping Liu[a], Fangfang Wang[a], Na Na[a], Jin Ouyang[a,*]

[a] College of Chemistry, Beijing Normal University, Beijing 100875, China
[b] Wuhan Centers For Disease Prevention & Control, Wuhan 430015, China

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ABSTRACT

A new and fast method for haptoglobin phenotyping was developed based on microchip electrophoresis with laser induced fluorescence detection. Haptoglobin phenotypes 1-1 and 2-2 were labeled with fluorescein isothiocyanate. The analyses were performed on glass microchip which was simply treated with sodium dodecyl sulfate. After the optimization of the separation conditions, Hp 1-1 and Hp 2-2 could be differentiated in 150 s and the detection limits for Hp 1-1 and Hp 2-2 were 0.39 and 0.62 μg/mL, respectively. Finally, the method was applied to human serum samples from healthy people and liver cancer patients. A decrease in Hp concentration for liver cancer patients was confirmed. Featuring high efficiency, speed, simplicity, the method reveals great potentials for the diagnosis of diseases and proteome research.

1. Introduction

Over the past decade, microchip electrophoresis (MCE), as a further miniaturized version of CE, has been developed dramatically owing to the potentials of its shorter analysis time, lower sample and solvent consumption (lower cost), smaller device size and more ease of integration and automation compared to liquid chromatography (LC) and CE [1–3]. Therefore, it promises to have a significant impact on almost all areas of biochemical and chemical analysis such as drug, DNA, chiral, single-cell, *etc.* [4–12]. Microchips for the analysis and separation of proteins have advanced tremendously in recent years. Proteins, a kind of biomacromolecules, particularly tend to surface adsorption in polymer-based chips, as well as on glass or silicon chips. Adsorption can interfere with microfluidic transport and can decrease separation efficiency and detection sensitivity. So surface coating is one of the critical techniques for the separation of protein. Wu and coworkers reported grafting epoxy-modified hydrophilic polymers onto poly(dimethylsiloxane) microfluidic chip to resist nonspecific protein adsorption [13]. Size-based protein separation was performed with an acid-labile surfactant (ALS) on a glass microchip dynamically coated with poly-(N-hydroxyethylacrylamide) polymer [14]. Among the several detection techniques employed in microchip, laser induced fluorescence (LIF) detection has become the most important detection technique because of its high sensitivity and low background noise. It is very important for the separation of proteins that microchip electrophoresis combine with laser induced fluorescence detection.

Haptoglobin (Hp) is an acute-phase plasma protein which plays an important part in binding and transporting of hemoglobin (Hb). In human serum, it is characterized by a genetic polymorphism with three structurally different phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2). Hp composed of two different polypeptide chains, the α-chain and the β-chain. The β-chain (40 kDa) is heavier than the α-chain and is identical in all Hp types. The Hp polymorphism results from variant α-chains. Hp 1-1 expresses only α^1-chains (8.9 kDa) and is an 98 kDa small molecule of homodimer or $(\alpha^1\beta)_2$, whereas α^2-chains (16 kDa) are present in Hp 2-1 and Hp 2-2 which show a range of moleculer mass (Hp 2-1: 86–300 kDa; Hp 2-2: 170–900 kDa) due to the existence of polymeric forms. Hp 2-1 is comprised of multiple heteropolymer forms including homodimer $(\alpha^1\beta)_2$, trimer $[(\alpha^1\beta)_2+(\alpha^2\beta)]$, tetramer $[(\alpha^1\beta)_2+(\alpha^2\beta)_2]$ and other linear polymers $[(\alpha^1\beta)_2+(\alpha^2\beta)_n]$. Hp 2-2 consists of trimer $(\alpha^2\beta)_3$, tetramer $(\alpha^2\beta)_4$ and other cyclic homopolymers $(\alpha^2\beta)_n$ [15]. Hp phenotypes show important functional differences, which have potential association with major diseases. A higher prevalence of coronary artery lesions and peripheral arterial occlusive lesions is observed among hypertensives with an Hp 2-2 phenotype, which have more severe myocardial infarctions and are at higher risk of developing refractory hypertension [16–18]. Furthermore, it can be observed that schizophrenia occurs more often in Hp 2-2 phenotype carriers [19]. In cervical cancer and cervical intraepithelial neoplasia (CIN), Hp 1-1 is a risk factor while Hp 2-2 appears protective [20,21]. As a result, it is imperative to have a suitable method for Hp phenotyping.

Current methods for the determination of Hp phenotypes are based on electrophoretic separation in sieving gels (starch, agarose

* Corresponding author at: College of Chemistry, No. 44, Beijing Normal University, Beijing 100875, China. Tel.: +86 10 58805373; fax: +86 10 62799838.
E-mail address: jinoyang@bnu.edu.cn (J. Ouyang).

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or polyacrylamide gel) and isoelectric focusing. Historically, starch and agarose gel electrophoresis for Hp phenotyping had been outdated due to low sensitivity and poor selectivity. In this regard, polyacrylamide gel electrophoresis (PAGE) has become one of the most important methods [22–26]. After separation, the proteins present in the gel can be detected by several methods such as the Coomassie Brilliant Blue (CBB) staining, silver-staining, chemiluminescent (CL) imaging, and so on. Though the detection technology of polyacrylamide gel electrophoresis has been continuously improved, this method is still time-consuming, labor-intensive, and semi-quantitative. In addition, high pressure gel permeation chromatography and mass spectrometry have also been used to differentiate Hp phenotypes [27–29]. Due to the superior separation efficiency and speed, capillary electrophoresis (CE) can rapidly differentiate Hp phenotypes [30–32]. The majority of commercially available CE instruments employ UV-absorbance detector, however, the limited optical path lengths result in low detection sensitivity.

In the present work, possibilities for a faster Hp phenotyping using glass MCE were investigated. In order to carry out the detection on MCE-LIF, fluorescein isothiocyanate (FITC) was chosen as precolumn derivatization reagent because of excellent fluorescence property, with which we can obtain high detection sensitivity. In addition, we used sodium dodecyl sulfate (SDS) solution to modify the microchannels and suppress the adsorption of proteins. As a result, strong Hp adsorption on the glass chip could be largely reduced. After conditions optimization, Hp 1-1 and Hp 2-2 could be differentiated by MCE. Compared with gel electrophoresis (about one to two days) [23] and conventional CE (about 400 s) [32], this analysis approach shows a significant advantage of taking less than 150 s. Meanwhile, lower concentration of Hp could be detected (0.39 μg/mL of Hp 1-1 and 0.62 μg/mL of Hp 2-2) with this method, while the detection limits for Hp 1-1 and Hp 2-2 were 40.0 and 31.3 μg/mL based on CE, respectively [32]. Lastly, this method shows other significant advantages of lower sample and solvent consumption (lower cost), smaller device size and more ease of automation compared with gel electrophoresis and conventional CE [1–3]. The paper therefore represents a significant step forward in the use of glass chip for rapid and sensitive analysis of Hp in serum, and for Hp depletion monitoring in liver cancer patients.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade or better and distilled water was used throughout. Haptoglobins (Hp 1-1, 98 kDa and Hp 2-2, 170–900 kDa) and FITC were purchased from Sigma (St. Louis, MO, USA). Serum samples of normal persons were obtained from the Affiliated Hospital of the Beijing Normal University. Serum samples of liver cancer patients were obtained from the Beijing Tumour Hospital. Sodium dodecyl sulfate (SDS) was obtained from Sino-American Biotechnology Co. (Beijing, China). Boric acid, sodium bicarbonate and sodium hydroxide were purchased from Beijing Chemical Plant (Beijing, China).

2.2. Solutions and sample preparation

Standard stock solutions (Hp 1-1 and Hp 2-2) containing 1 mg/mL of each analyte were prepared by dissolving precisely weighed standard sample in ultrapure water and stored at −20 °C. The serum samples from healthy people and patients were treated with the Aurum Serum Protein Mini Kit (Biorad, USA) to remove both HSA and immunoglobulin (IgG). Then, HSA and IgG depleted sera were stored at −20 °C. 1 mg of FITC was dissolved in 50 μL DMSO and 950 μL actone to yield a stock solution (1 mg/mL). For labeling Hp, 100 mM carbonate buffer (pH 9.31) was used. A solution of 100 mM boric acid (pH 10.56) was used as running buffer for the separation of FITC-labeled Hp 1-1 and Hp 2-2. All solutions were filtered through 0.22 μm membrane filters before use. Ultrapure water (resistivity 18.2 MΩ cm; Barnstead, Dubuque, IA, USA) was used for preparation of all the aqueous solutions.

2.3. Derivatization procedure

Hp or serum was labeled with FITC as follows:

After 5 μL Hp solution was mixed in 11 μL of 100 mM carbonate buffer (pH 9.31), 9 μL FITC, which was prepared by diluting the stock solution of FITC with 100 mM carbonate buffer (1:100), was added. Typically, the mixed solution was reacted in the dark overnight at room temperature. Prior to analysis, the derivatization solution was stored in the dark at 4 °C.

The derivatization procedure of the serum samples removed both HSA and IgG is the same as the standard's.

2.4. Apparatus

The experiments were performed on the Microfluidic Apparatus (Hang Zhou City Hua Zhong Science and Technology Ltd., China) equipped with a laser-induced fluorescence (LIF) detector (excitation wavelength at 488 nm, emission wavelength at 520 nm). The structure of the glass microchip is illustrated in Fig. S-1. The length of sample injection channel between the sample reservoir (S) and the sample waste (SW) was 1 cm, while the total separation length between the buffer reservoir (B) and the buffer waste (BW) was 5 cm and the effective separation length was 4 cm. The channels dimensions were 50 μm in depth and 25 μm in width, respectively.

2.5. Microchip electrophoresis

Before the first use, the channels were first washed with HCl (10%) for 20 min, followed by a 30 min rinse with 2 M NaOH. Then 0.1 M NaOH, ultrapure water, 10% SDS, and the buffer were sequentially flushed through the channels for 30, 10, 30, 10 min, respectively. The processed channels can be recycled without any treatment unless different samples were injected. After the microchip was treated, the reservoirs B, SW, and BW were filled with the electrophoretic buffer, reservoir S was filled with sample solution. Then, the chip was placed onto the slab and the platinum electrodes were put into four reservoirs. Samples were introduced by a pinched injection and separated using the voltage program given in Table S-1. MCE were performed with a borate buffer solution (100 mM, pH 10.56).

3. Results and discussion

3.1. Modification of surface with SDS

Although MCE devices are widely used at present, separation of proteins in MCE still faces more challenges compared to CE because of the reduced separation length and high specific surface area. As a result of the high adsorptive interactions between proteins and glass surface induced by high specific surface area, MCE has a significant loss of separation efficiency and detection sensitivity. In order to eliminate the adsorption of analytes on the channel walls, the surface must be modified so as to decrease the peak broadening and increase the signal intensities [33,34].

The dynamic coating of SDS is an effective mean for both controlling electroosmotic flow (EOF) in the channels and preventing

protein adsorption on the channel walls. SDS belongs to a family of anionic surfactants and includes a long carbon chain (C-chain). When added to a sufficiently high concentration, the strongly hydrophobic C-chain will interact with the glass channels and the adsorption of analytes will be controlled. Before electrophoresis, the channels of microchip were treated by SDS for 30 min, followed by rinsing with buffer, which could improve the channels' hydrophilicity and reduce the strong adsorption.

The same sample of Hp-FITC was detected before and after the treatment of microchip, respectively. The results were shown in Fig. 1, coating the channel walls with SDS can suppress protein adsorption. Compared to direct electrophoresis analysis on the native glass microchip, the migration time and peak shape of Hp showed obvious differences while rinsing the channel with 10% SDS before sample injection and an improvement in sensitivity of at least 10-fold can be obtained. In Fig. 1A, broad and low peaks are produced due to adsorption on the microchip inner surface, while in Fig. 1B peaks are clearly observed due to surface modification of microchip with SDS. The result showed that the surfactant SDS could be used to dynamically modify the surface and prevented protein adsorption on the channel walls.

3.2. Separation and determination of Hp phenotypes

There is only one kind of phenotype (Hp 1-1, Hp 2-1, or Hp 2-2) exists in each human serum. Therefore, FITC was chosen as internal standard to differentiate phenotypes of Hp by comparing the relative retention times between FITC and each peak of Hp phenotype. The electropherograms for Hp 1-1 and Hp 2-2 with fluorescent detection on the dynamic SDS-coated glass chip are shown in Fig. 2. The running buffer was 100 mM borate buffer containing 5 mM SDS, adjusted to pH 10.56 using 0.1 M NaOH. The result showed that two phenotypes were absolutely baseline-resolved. Likewise, from the electropherogram it can be observed that two phenotypes have been differentiated by the fact that Hp 2-2 migrates faster than Hp 1-1, with migration times of 110 s and 125 s, respectively.

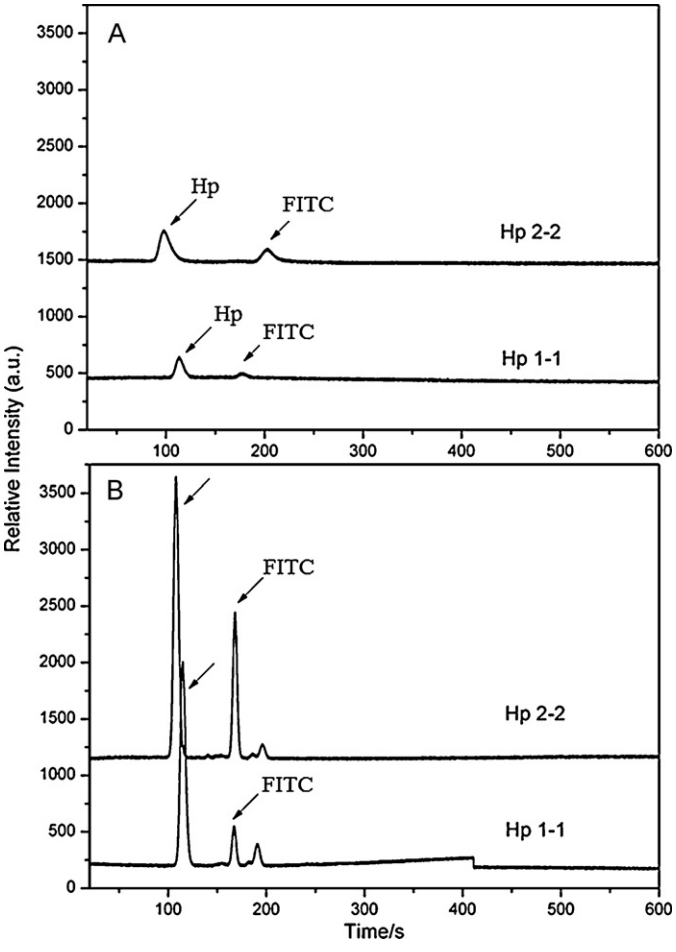

Fig. 1. Electropherograms of the separation of FITC-labeled Hp 1-1 and Hp 2-2: (A) native glass chip; (B) SDS modified glass chip. Separation conditions: 100 mM borate buffer; pH 10.56; separation voltage 800 V; 5 cm separation length; 4 cm detection length.

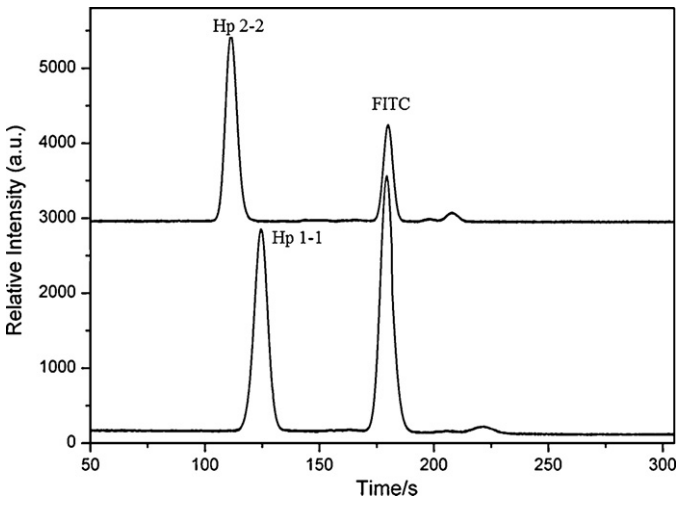

Fig. 2. Electropherograms of the separation of FITC-labeled Hp 1-1 and Hp 2-2 on the SDS modified glass chip. Separation conditions: 100 mM borate buffer; pH 10.56; 5 mM SDS, separation voltage 800 V; 5 cm separation length; 4 cm detection length.

3.3. Optimization of separation condition

In order to obtain the optimum conditions for separation and determination of Hp phenotypes, some preliminary experiments were performed. Standard sample of FITC-labeled Hp 1-1 and Hp 2-2 was injected in the microchip with different experiment conditions involving concentration of electrolyte, pH of running buffer, SDS concentration and applied voltage.

In this study, the borate buffer was used as background electrolyte for the separation of FITC-labeled Hp 1-1 and Hp 2-2, the pH value of borate buffer can influence the velocity of EOF, protein mobility and protein adsorption on the walls by altering the charges of the proteins and the channel walls. Therefore, the effects of pH ranged from 8.50 to 11.50 on the separation of FITC-labeled Hp 1-1 and Hp 2-2 were mainly tested. The results showed that the pH influenced the separation efficiency of Hp 1-1 and Hp 2-2 (Fig. 3). From the electropherogram it can be seen that pH 10.56 is the best condition of separation. Further increasing pH values may result in excessively long migration times, less reproducible electroosmotic flow and reduced fluorescence emission of the protein–FITC complex, and so was not a suitable alternative.

In addition, the concentration of borate buffer got optimized. As we all know, increasing the concentration of the separation buffer results in decrease in electroosmotic mobility, which should increase the opportunity for complete resolution of the proteins by providing more time. As a result, the effect of borate buffer concentration on the separation was investigated from 50 to 120 mM and set at 100 mM for the separation concentration.

The applied voltage on separation was also optimized from 500 to 2000 V. The results indicated that the migration times and the

Table 1
Detection limits, linearity and repeatability.

Analyte	Equation for calibration curves	Linear range ($\mu g\,mL^{-1}$)	R^2	Detection limit ($\mu g\,mL^{-1}$) (S/N = 3)
Hp 1-1	$y = 2.967x + 0.205$	0.39–200	0.9923	0.39
Hp 2-2	$y = 1.989x + 2.426$	0.62–200	0.9915	0.62

Separation conditions: pH 10.56, separation voltage 800 V, buffer solution of 100 mM borate (containing 5 mM SDS), injection time: 10 s.

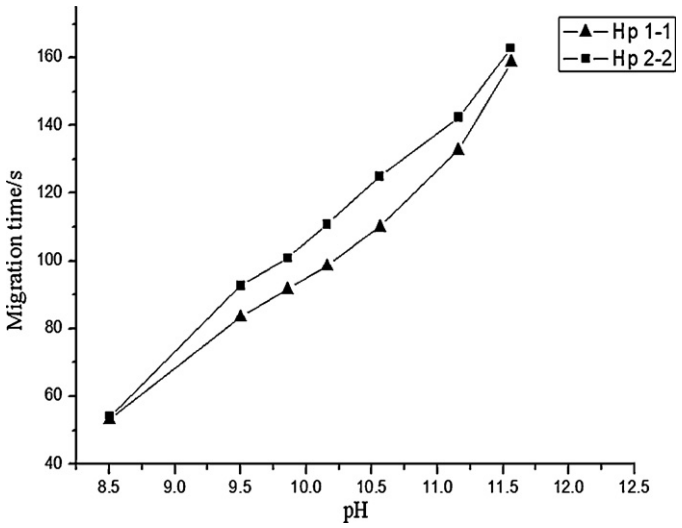

Fig. 3. Effect of buffer solution pH on the separation of FITC-labeled Hp 1-1 and Hp 2-2 on the glass chip. Other conditions as provided in Fig. 2.

separation efficiency of FITC-labeled Hp1-1 and Hp2-2 decreased with the increase of voltage. Considering both the analysis time and the resolution, a separation voltage of 800 V was considered optimal.

The concentration of SDS in running buffer was also investigated, and it was optimized in running buffer. The concentration of 5 mM gave the best performances for each peak and the optimal resolution.

3.4. Quantitative analysis of Hp

The separation reproducibility on the modified microchip was shown in Fig. 4, and is quoted in terms of percentage relative standard deviation (% RSD) of the migration times and the peak height. In the channels, 4 successive injections of Hp in 100 mM borate buffer showed that run-to-run RSD for migration time is 1.2%, and for peak height less than 3.1%. The detection limits for Hp 1-1 and Hp 2-2 were calculated using serial dilution of the stock solutions, and the results in Table 1 are based on a minimum signal-to-noise ratio of three. Results indicate that the detectable concentrations were 0.39 μg/mL for Hp 1-1 and 0.62 μg/mL for Hp 2-2.

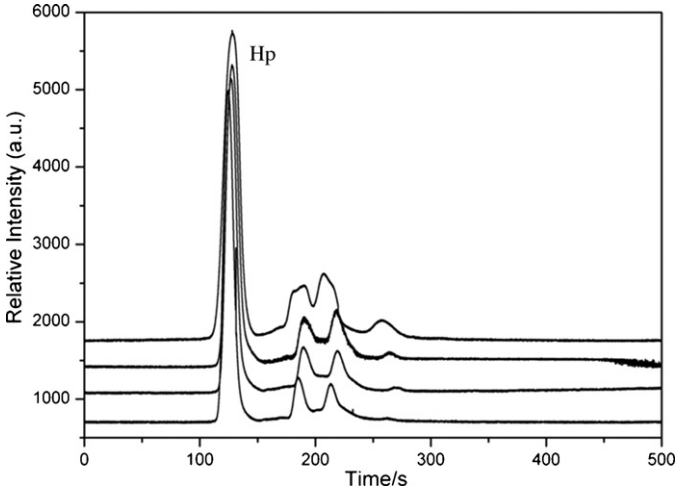

Fig. 4. Reproducibility of the separation of FITC-labeled Hp 2-2 on the SDS modified glass chip. Other conditions as provided in Fig. 2.

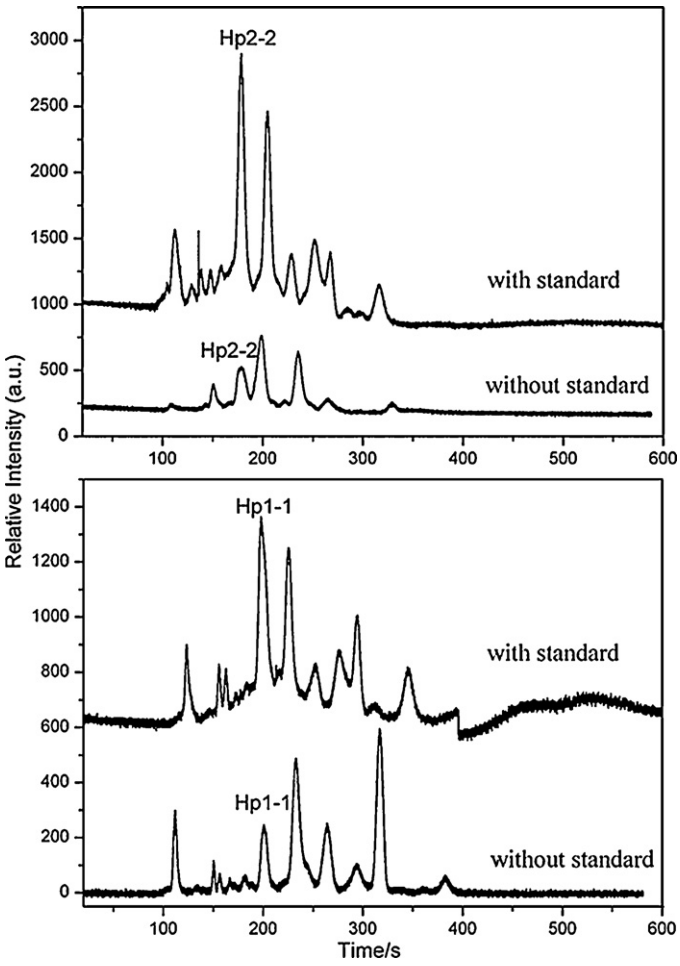

Fig. 5. Electropherograms of healthy subject's serum sample with and without the addition of standard on the SDS modified glass chip. Samples were diluted 7 times. Separation conditions: 100 mM borate buffer, pH 10.56; 5 mM SDS; separation voltage 800 V; 5 cm separation length; 4 cm detection length.

3.5. Application

Since MCE is simple, fast, and high throughput compared with traditional CE, we suggested the applicability for the analysis of human serum sample and the diagnosis of diseases. However, HSA and IgG, which are the most abundant in human serum, interfere with the detection of other proteins. These two proteins were removed from the human serum using the aurum serum protein

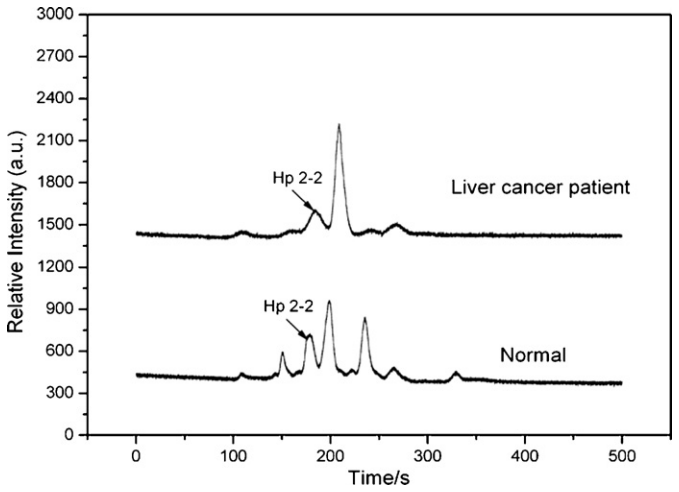

Fig. 6. Comparison of healthy subject's and liver cancer's serum sample which were diluted 7 times. Other conditions as those provided in Fig. 5.

mini kit. HSA and IgG depleted sera with the derivatization by FITC were then injected into the microchip. Fig. 5 shows the electropherograms of Hp 1-1 and Hp 2-2 individual serum samples and peaks of Hp were identified by the addition of standards. The electropherogram appears that Hp 1-1 and Hp 2-2 individuals could be differentiated by the different migration times. It was also noted that Hp in the serum migrated much slower than the standard samples. It was known that other proteins in the serum, such as transferrin, fibrinogen, complement C3, would play a role as matrix to affect the migration of Hp. In addition, serum samples diluted by commercial binding buffer take a higher ionic strength, resulting in slower apparent mobilities for the proteins.

There has been evidence indicating that serum Hp may be a potential biomarker in the diagnosis of liver cancer disease [35]. Serum samples from liver cancer patients were analysed to investigate the feasibility of the proposed method. At the same time, the result was compared with that obtained by the method based on PAGE. The comparison between serum samples from three liver cancer patients and five healthy people were carried out to find the difference. All samples from patients and healthy subjects were processed and injected under the same experimental conditions. Representative electropherograms of liver cancer's serum and a healthy individual are shown in Fig. 6. It is observed that there is a decrease of Hp concentration in liver cancer patients. The result was in accordance with that obtained by the method of PAGE (shown in Fig. S-2). The observation of low levels of Hp in liver cancer could be attributed to the damage of liver which results in a decrease of protein synthesis. Meanwhile, the MCE-LIF detection clearly improved the sensitivity of Hp detection. The literature concentration of Hp in serum samples was calculated by the relative hemoglobin binding capacity (HBC) of the various Hp phenotypes which were based on earlier immunodiffusion methods [36,37]. In these methods, serum Hp concentration was underestimated because the diffusion of serum Hp immune complexes is impaired in gels (typically in the analysis of polymeric Hp 2-2). In view of the lower reference range for Hp 2-2 (0.38–1.5 g/L) [15], the sensitive MCE-LIF detection is particularly important in detecting Hp 2-2 of liver cancer patients and healthy subjects. Therefore MCE can be a promise method which is used in the diagnosis of liver cancer and other major diseases.

4. Conclusion

In summary, the MCE-LIF method proposed in this work has been developed for fast Hp phenotyping. Under the optimum conditions, Hp phenotypes (Hp 1-1 and Hp 2-2) could be differentiated in 150 s. The separation speed of MCE is faster than that of CE which required nearly 400 s. Meanwhile, the described assay has proven to be useful for the determination of Hp phenotype in serum samples and detecting Hp decreases in liver cancer patients. Low running cost, short separation time, and sufficient sensitivity are the important features of the method. The work clearly indicates that the MCE-LIF method may be a powerful tool for clinical and routine analysis of haptoglobin phenotypes in human serum, which will provide a novel diagnoses method for liver cancer disease and other diseases related to haptoglobin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.03.066.

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