Yuanhong Xu
Jing Li
Erkang Wang

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Changchun, Jilin, P. R. China

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Research Article

Sensitive, label-free protein assay using 1-ethyl-3-methylimidazolium tetrafluoroborate-supported microchip electrophoresis with laser-induced fluorescence detection

Based on the dimer–monomer equilibrium movement of the fluorescent dye Pyronin Y (PY), a rapid, simple, highly sensitive, label-free method for protein detection was developed by microchip electrophoresis with LIF detection. PY formed a nonfluorescent dimer induced by the premicellar aggregation of an anionic surfactant, SDS, however, the fluorescence intensity of the system increased dramatically when proteins such as BSA, bovine hemoglobin, cytochrome *c*, and trypsin were added to the solution due to the transition of dimer to fluorescent monomer. Furthermore, 1-ethyl-3-methylimidazolium tetrafluoroborate ($EMImBF_4$) instead of PBS was applied as running buffers in microchip electrophoresis. Due to the excellent properties of $EMImBF_4$, not only nonspecific protein adsorption was more efficiently suppressed, but also approximately ten-fold higher fluorescence intensity enhancement was obtained than that using PBS. Under the optimal conditions, detection limits for BSA, bovine hemoglobin, cytochrome *c*, and trypsin were 1.00×10^{-6}, 2×10^{-6}, 7×10^{-7}, and 5×10^{-7} mg/mL, respectively. Thus, without covalent modification of the protein, a protein assay method with high sensitivity was achieved on microchips.

Keywords:
Ionic liquid / Label-free protein assay / Microchip / Protein
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1 Introduction

Proteins play essential roles in biochemical systems and study of their rapid, sensitive detection and quantification remains a great challenge that attracts lots of attention worldwide [1]. One of the most promising methods of protein detection is the utilization of microfluidic systems, such as microchip electrophoresis, due to their excellent properties such as easy integration, speed of analysis, lower reagent consumption, reduced waste, and portability [2–4]. Among the several detection techniques employed in microchip analysis, LIF detection method is most easily adapted to the dimensions of microchips [5]. The coherence and low divergence of a laser beam make it easy to focus on very small analyte volumes and obtain much high irradiation, resulting in one of the most sensitive and powerful means of any detection systems [5, 6]. However, only a few compounds exhibit native fluorescence and pre- or postcolumn labeling with fluorescent markers is required for protein analysis [5]. Although labels have significantly improved protein research, they are not without disadvantages [7, 8]. For example, the labeling efficiency varies with different proteins and leads to difficulty in quantifying the detection accurately; labels hinder the natural behavior and alter the electrophoretic mobility of proteins; labeling is a time-consuming, complex process and with relatively high costs. These limitations have motivated the developments of procedures for label-free or unlabeled formats of protein detections in microfluidic systems [9–11]. Jin *et al.* [9] incorporated fluorescent intercalating dye into the separation medium and achieved the dynamic labeling microchip electrophoresis for LIF detection of protein–SDS complexes without pre- or

Correspondence: Professor Erkang Wang, State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Changchun, Jilin 130022, P. R. China
E-mail: ekwang@ciac.jl.cn
Fax: +86-431-85689711

Abbreviations: $EMImBF_4$, 1-ethyl-3-methylimidazolium tetrafluoroborate; **IL,** ionic liquid; **PBS,** phosphate buffer solution; **PY,** Pyronin Y

postcolumn labeling. Hellmich *et al*. [10, 11] demonstrated a single cell manipulation, analytics, and label-free protein detection in PDMS microfluidic devices for systems nanobiology. Despite all the contributions, it is still urgent to develop other simple, more sensitive, label-free LIF technique for protein determination on microchips.

In addition, proteins separation in microchannels is still a focusing topic of intensive biological research and a challenge because of their adsorption onto the surface of the microchips such as PDMS ones [12, 13]. Various methods [14–18] have been taken to either chemically or physically modify the surface of PDMS microchannels. There is hardly a single method suitable for all protein analysis problems. Dynamic coating is the easiest and most convenient way for surface modification [14], in which surface-active compounds like polymers or surfactants dissolved in running buffers, the hydrophobic tail can easily adsorb to hydrophobic PDMS surfaces, controlling the EOF depending on the charge of the modifying compounds and reducing the analytes adsorption. However, for pure surfactants used as additives of common running buffer, the capability of resolving analyte adsorption to PDMS surface was limited. With the fast development of ionic liquids (ILs) for a number of applications [19, 20], they also penetrated into the field of separation science and technology, such as being used as supporting electrolytes or additives to running buffers in standard CE [21, 22]. Our previous work [23] has investigated the effects of ILs used as supporting electrolytes of microchip electrophoresis, and hybrid coating with IL and nonionic surfactant for more efficient PDMS modification was confirmed. However, longer separation time was needed for the labeled protein analysis. In addition, the synergistic effect between IL and other ionic surfactants such as the most popular anionic surfactant, SDS, which has not been investigated and may show more functions on microchip separations.

Our work was inspired by the fact that induced by the premicellar aggregation of an anionic surfactant, cationic dyes can form the rarely weak fluorescent dimer, whereas when proteins are added into the dye–surfactant system, some dye dimers turn into monomer, resulting in the fluorescence enhancement of the dye–surfactant system [24–26]. Conventional fluorescent methods cannot discriminate between specific fluorescence signals and nonspecific background signals and result in the high detection limit and low sensitivity of protein determination [24–26]. To the best of our knowledge, introduction of this principle into microfluidic applications was never reported.

Herein, based on the above principle of the monomer–dimer equilibrium of the fluorescent dye Pyronin Y (PY), in the presence of the anionic surfactant SDS, BSA was chosen as the representative of proteins, a novel label-free protein assay method was conducted by the microchip separation combined with LIF detection technique. In order to keep consistent with the used surfactant in the sample preparation, SDS was also used in the running buffer. To completely overcome the protein adsorption in the microchannels, the IL 1-ethyl-3-methylimidazolium tetrafluoroborate ($EMImBF_4$) was directly applied as the supporting electrolytes and optimization experiments were performed to obtain the highest protein detection sensitivity. This method not only avoided tedious labeling steps relating to covalent modification of the proteins but also still further enhanced and expanded the inherent properties of microchip combining with the sensitivity of LIF detection. Finally it reached simple, quick, and high-sensitive label-free protein detection on microchips.

2 Materials and methods

2.1 Chemicals and materials

PY and SDS were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sylgard 184 PDMS was obtained from Dow Corning (Midland, MI, USA). BSA was purchased from Pierce Biotechnology (Rockford, IL, USA). The IL $EMImBF_4$ used in this work was obtained from the Centre for Green Chemistry and Catalysis, Lanzhou Institute of Chemical Physics. It was synthesized following the procedure described elsewhere [27]. The starting materials were obtained from Sigma–Aldrich (Steinheim, Germany). All chemicals were of analytical reagent grade and used as received without further purification. All solutions were prepared with deionized water, which was processed with Milli-Q ultrahigh purity water system (Millipore, Bedford, MA, USA). Phosphate buffer solutions (PBS, pH 7.5) were prepared from stock solution of 100 mM and stored at 4°C. The surfactant of SDS was prepared freshly, and the stock concentration was 50 mM. The CMC is approximately 8 mM at 25°C. PY stock solution of 1 mM was stored at 4°C. BSA (5 mg/mL) stock solution was prepared in 10 mM PBS (pH 7.5). A series of BSA solutions for calibration curve, reproducibility, LOD, and other parameters at different electrophoresis conditions were prepared by diluting the stock solution with 10 mM PBS (pH 7.5) and adding equivalent concentrations of SDS and PY.

2.2 Electrophoresis procedure on PDMS/glass chip

Fabrication of PDMS/glass chip was designed and fabricated as described previously [28]. In brief, PDMS microchannels were got from molding on lithographically and wet chemically prepared glass master. The PDMS replica with cross-electrophoresis channel had the feature of 60 μm wide and 10 μm deep. After the PDMS replica was created with four reservoirs at each end of the crosschannel using a hole punch, a hybrid chip was created through sealing of the PDMS slab to a flat glass. Electrophoresis is carried out in a "T" microfluidic chip (Fig. 1A) and the electrophoresis procedure was designed and performed as published elsewhere [29]. Prior to electrophoresis, the channels of the hybrid chip were flushed with Milli-Q water, 0.1 M NaOH, and Milli-Q

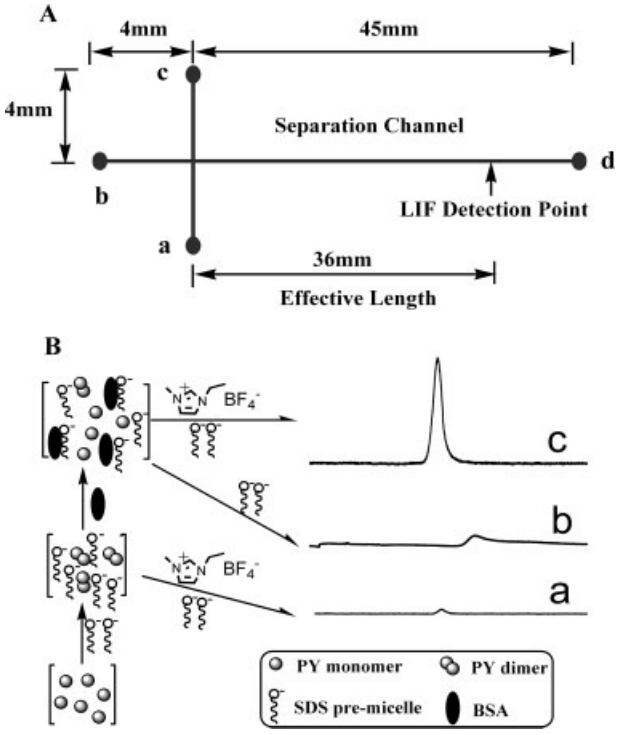

Figure 1. (A) Layout of the microfluidic chip and (B) schematic illustration of label-free protein assay by the IL-assisted microchip electrophoresis with LIF detection.

water, respectively, for 10 min, equilibrated with the running buffer for 15–20 min. These solutions were directly infused into the reservoirs, then pumped through channels by a vacuum air pump. The sample was placed in reservoir A, while reservoirs B–D were filled with the running buffers. Platinum electrodes were inserted into the reservoirs to apply voltages for electrophoresis. Two sets of voltages were applied for sample loading and electrophoretic separation: (i) $V_a = 1.20$ kV, V_d = ground for 10 s and (ii) $V_a = 1.05$ kV, $V_c = 1.05$ kV, $V_b = 1.20$ kV, V_d = ground for appropriate time. All experiments were performed on the Micralyne Microfluidic Tool Kit (μTK) (Micralyne, Edmonton, Alta., Canada). LIF detection was carried out at the detection point using the 532 nm ν-doubled Nd–YAG laser (4 mW). The luminescent confocal detection module employed a 40×0.55 NA aspherical lens to focus the beam onto the channel of the chip. The optical path into the PMT has a 10 nm bandpass filter centered at 568.2 nm. Control and data collection were performed with a LabView software (National Instruments, Austin, TX). In the experiments the acquisition frequency of the PMT was 25 Hz and the gain was 0.400.

2.3 Fluorescence spectra

Fluorescence spectra were collected with an LS55 Luminescence Spectrometer (Perkin-Elmer Instruments, UK). For recording the excitation spectrum, the emission wavelength was set at 568 nm with spectral bandwidth (10 nm) while the excitation wavelength was scanned at a specified scan rate from 250 to 550 nm. For recording the emission spectra, the excitation wavelength was set at 532 nm with spectral bandwidth (10 nm) while the excitation wavelength was scanned at a specified scan rate from 555 to 800 nm. Solutions of 5×10^{-4} or 0.05 mg/mL BSA in 10 mM PBS (pH 7.5) and 5 mM SDS were prepared, respectively, for the fluorescence studies.

3 Results and discussion

3.1 Mechanism of the label-free protein assay and the background experiment

In this experiment, PY was chosen as the representative of cationic dyes that can form monomer–dimer in aqueous solution. According to a previous report [30], when the dye concentration used was about 5 μM, the dye existed mainly in the monomer form, only very small amounts of dimer were present, thus 5 μM PY was finally accepted as the appropriate dye concentration for further experiments. As can be seen in the schematic diagram (Fig. 1B), when SDS was added into the dye solution, the dimerization occurred and a higher amount of dimer was obtained, which was nonfluorescent. Accompanying the addition of protein BSA to the dye–surfactant system, BSA and SDS can interact and form negative micelle-like cluster complex, which destroys the microenvironment of PY dimer formation and makes main PY dimers turn into monomer [31]. The monomer–dimer transition was manifested by the dramatic fluorescence intensity change in the electropherograms of microchip electrophoresis with LIF detection. Figures 1B-a and c exhibit the electropherograms of 5 μM PY containing 10 mM PBS and 5 mM SDS in the absence and presence of BSA, respectively, when 5 mM SDS in 10 mM $EMImBF_4$ was applied as running buffers. For the same concentration of BSA in 5 μM PY containing 10 mM PBS and 5 mM SDS, lower fluorescence intensity and an unsymmetrical peak of PY were obtained when 5 mM SDS in 10 mM PBS was used as running buffer. Subsequently, quantification of BSA without being fluorescently labeled can be achieved on $EMImBF_4$-assisted microchips according to the linear dependence of PY fluorescence intensity enhancement on BSA concentration.

Background experiments were done to avoid the interference of background fluorescence. Figures 2A and B are the excitation spectra and emission spectra of 5×10^{-4} mg/mL BSA in 10 mM PBS (pH 7.5) and 5 mM SDS, respectively. It showed that the maximum excitation wavelength was 280 nm (Fig. 2A) and no emission fluorescence when the excitation wavelength was set at 532 nm (Fig. 2B) for BSA in the experiment conditions. In addition, fluorescence spectra for 0.05 mg/mL BSA were also conducted under the same conditions and the similar phenomenon was obtained.

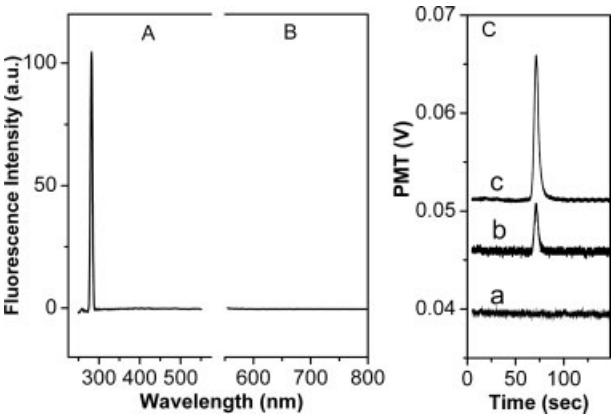

Figure 2. (A) Excitation spectra of 5×10^{-4} mg/mL BSA in 10 mM PBS (pH 7.5) and 5 mM SDS; the emission wavelength was set at 568 nm with spectral bandwidth (10 nm) while the excitation wavelength was scanned at a specified scan rate from 250 to 550 nm. (B) Emission spectra of 5×10^{-4} mg/mL BSA in 10 mM PBS (pH 7.5) and 5 mM SDS; the excitation wavelength was set at 532 nm with spectral bandwidth (10 nm) while the excitation wavelength was scanned at a specified scan rate from 555 to 800 nm. (C) Electropherograms of (a) 5×10^{-4} mg/mL BSA in 10 mM PBS (pH 7.5) and 5 mM SDS; (b) 5 μM PY in 10 mM PBS (pH 7.5) and 5 mM SDS; (c) 10 μM PY in 10 mM PBS (pH 7.5) and 5 mM SDS. Running buffer: 10 mM $EMImBF_4$ containing 5 mM SDS. The effective length of the separation channel was 3.6 cm.

Also as can be seen in Fig. 2C-a, no fluorescence intensity was observed for 5×10^{-4} mg/mL BSA in 10 mM PBS (pH 7.5) containing 5 mM SDS using the microchip electrophoresis with LIF detection. Figures 2C-b and c represent the electropherograms of 5 μM, 10 μM PY in 10 mM PBS containing 5 mM SDS, respectively. The peak of PY fluorescence was confirmed as the fluorescence intensities increased with the increasing PY concentrations. Together with the above results, it can be sure that no background disturbance from the BSA-surfactant system was produced for PY detection and the feasibility of this label-free detection method can be ensured.

3.2 Effects of $EMImBF_4$ used as supporting electrolytes

The IL $EMImBF_4$ chosen here possessed excellent characteristics, such as high conductivity of 14 mS/cm, low viscosity of 34 mPa·s at 25°C [32], miscibility with water and good solvating properties, and qualified it as one of the suitable supporting electrolytes for microchip electrophoresis [23]. Thus, the synergistic effects between $EMImBF_4$ and SDS were investigated in detail in further experiments. Combining with appropriate concentrations of SDS, $EMImBF_4$ was directly used as the supporting electrolyte without adding other buffers to adjust its pH values.

To characterize the positive effects of $EMImBF_4$ solution used as running buffer, PBS and $EMImBF_4$ at the equivalent concentration of 10 mM were prepared, both containing 5 mM SDS. With the same sample injections, a smaller background current and shorter migration time were achieved for $EMImBF_4$ than that for PBS. It showed that $EMImBF_4$ not only had the ability to separate hydrophobic analytes while maintaining an adequate background current, but also produced smaller Joule heating in the microchannels, which induced more rapid sample analysis and better reproducibility. To confirm this hypothesis, further comparisons were conducted. Same samples of 7×10^{-5} mg/mL BSA–5 μM PY–5 mM SDS in 10 mM PBS (pH 7.5) were prepared and injected consecutively twice, respectively. For running buffer of 10 mM PBS containing 5 mM SDS, the electropherogram of the first injection was difficult to recover back to baseline after the appearance of the PY peak (Fig. 3A-a), thus in the subsequent injection, unstable baseline and a broader, lower PY peak were presented (Fig. 3A-b). That is to say, SDS can be used as surface modifier of the PDMS surface, the small hydrophobic molecule adsorption on PDMS surface can be efficiently minimized, however, certain difficulties may be encountered for macromolecules such as proteins. Accordingly, nonspecific adsorption of proteins in the PDMS microchannels occurred. Although BSA was nonfluorescent under this condition, the properties of the microchannel were influenced and the baseline got unstable, not to mention the subsequent injections and reproducibility of this method. Conceivably, the solution of 5 mM SDS in 10 mM PBS was not perfect and applicable for protein analysis herein. Meanwhile, for the running buffer of 10 mM $EMImBF_4$ containing 5 mM SDS, the migration time, peak shape, and height were reproducible, and the baseline maintained stable for the successive injections (Figs. 3B-a' and b'). It clearly indicated that the nonspecific protein adsorption to PDMS surface can be completely suppressed

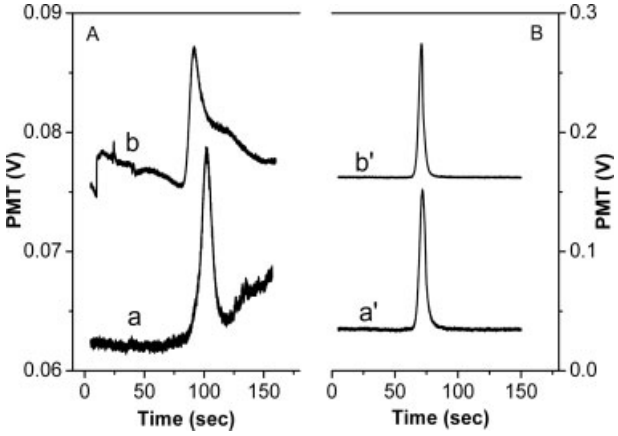

Figure 3. Effects of IL used as supporting electrolytes. Electropherograms of (A) (a) the first time and (b) the second time of sample injection using 10 mM PBS containing 5 mM SDS as running buffers; (B) (a') the first time and (b') the second time of sample injection using 10 mM $EMImBF_4$ containing 5 mM SDS as running buffers. Sample: 7×10^{-5} mg/mL BSA–5 μM PY–5 mM SDS in 10 mM PBS (pH 7.5). The effective length of the separation channel was 3.6 cm.

when using 10 mM $EMImBF_4$ containing 5 mM SDS as running buffer. Thus, easily understood, due to the incomplete suppression of protein adsorption to PDMS surface using 10 mM PBS as supporting electrolytes, the effective sample injection quality was reduced and lower fluorescence intensity was obtained. Meanwhile, the protein adsorption to the PDMS surface was completely suppressed using 10 mM $EMImBF_4$ as supporting electrolyte, the effective sample injection quality was guaranteed and the fluorescence intensity using 10 mM $EMImBF_4$ as supporting electrolyte (Figs. 3B-a' and b') was eight- to ten-fold higher than that using 10 mM PBS (Figs. 3A-a and b). In other words, the capability of SDS for resolving protein adsorption in PDMS microchannels was boosted up and the fluorescence intensity was greatly enhanced in the presence of $EMImBF_4$. It again confirmed the excellent properties of $EMImBF_4$ used as supporting electrolyte, and was suitable for the establishment of this label-free protein detection method.

3.3 Method optimization

As mentioned above, 5 μM PY is the critical concentration at which the monomer form mainly exists and small amounts of dimer begin to appear [30]. Moreover, sample injection of 5 μM PY produced moderate fluorescence intensity – it was not very too high, but still distinguishable from the background in the electropherograms of microchip electrophoresis with LIF detection. Thus, 5 μM PY was chosen as the "blank" sample for further experiments (blank sample meant that no BSA was added into the PY-SDS system).

To further optimize the sample preparation, a constant addition of 7×10^{-5} mg/mL BSA to the 5 μM PY-SDS system, study of the influence of SDS concentration on the fluorescence intensity was carried out from 0 to 20 mM (see Fig 4). When the concentration of SDS was below 0.5 mM, no significant peaks were observed because the SDS concentration was too low to reduce the PY and protein adsorption in the microchannels. The fluorescence intensity of PY increased with increasing SDS concentration from 0.5 to 5 mM. As the SDS concentration was above 5 mM, the fluorescence intensity remained fairly unchangeable. The maximum increase in the fluorescence emission intensity was obtained in the presence of 5 mM SDS. Furthermore, the CMC of SDS is approximately 8 mM, thus 5 mM SDS was in agreement with the required premicellar aggregation concentration of the anionic surfactant, which induced the conversion of PY monomer to dimer. Thus, 5 μM PY −5 mM SDS in 10 mM PBS was selected as the optimized background system for BSA detection.

As mentioned above, the IL $EMImBF_4$ was selected as the BGE, then the effect of $EMImBF_4$ concentration on fluorescence intensity, migration time, *etc.* of protein assay was investigated. Easily understood, when the concentration was below 5 mM, unstable baseline and low fluorescence intensity were obtained due to the low ionic strength. Although a higher concentration can provide higher ionic strength and conductivity, it gave longer migration time and broader peaks ascribing to higher Joule heating in the microchannels resulting from higher electrophoretic current. When the best compromise between high ionic conductivity and electrophoretic current (approx. 5 μA in the separation channel) reached the concentration of 10 mM $EMImBF_4$, the highest fluorescence intensity, shorter migration time (approx. 71 s) and better reproducibility were obtained. To keep consistent with the surfactant concentration used in the sample solution, 5 mM SDS was also applied as the finally chosen additives of 10 mM $EMImBF_4$.

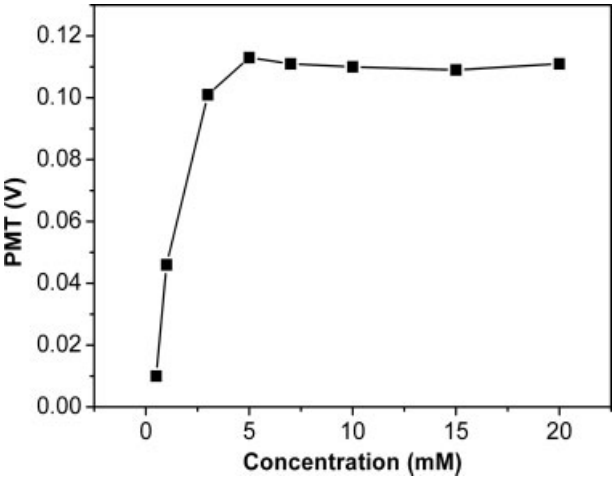

Figure 4. Effects of SDS concentration included in the sample on the fluorescence intensity enhancement of 7×10^{-5} mg/mL BSA–5 μM PY in 10 mM PBS (pH 7.5). Running buffer: 10 mM $EMImBF_4$ containing 5 mM SDS. The effective length of the separation channel was 3.6 cm.

Injection time and voltage were also optimized and the best compromise between sample loading and efficiency was achieved when two sets of voltages applied for sample loading and electrophoretic separation were set at (i) $V_a = 1.20$ kV, V_d = ground for 10 s and (ii) $V_a = 1.05$ kV, $V_c = 1.05$ kV, $V_b = 1.20$ kV, V_d = ground for appropriate time. In addition, the acquisition frequency of the PMT was set at 25 Hz, the gain was set at 0.400 and effective separation distance was 3.6 cm.

3.4 Repeatability, linearity, and detection limit of BSA

Under the optimized conditions as mentioned above, 7×10^{-5} mg/mL BSA–5 μM PY–5 mM SDS in 10 mM PBS (pH 7.5) was injected consecutively six times to determine the repeatability of fluorescence intensity based on peak height and migration time. RSDs of the fluorescence intensity and the migration time were 2.50 and 1.97%, respectively. The high reproducibility indicated that this approach can efficiently minimize the PY and BSA adsorption in the PDMS microchannels and was accurate for BSA detection.

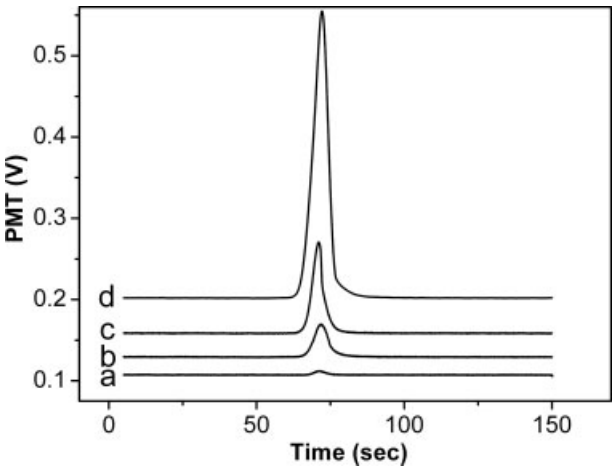

Figure 5. Electropherograms of (a) no BSA; (b) 1×10^{-5} mg/mL BSA; (c) 7×10^{-5} mg/mL BSA; (d) 5×10^{-4} mg/mL BSA included in 5 μM PY–5 mM SDS–10 mM PBS, respectively. Running buffer: 10 mM $EMImBF_4$ containing 5 mM SDS. The effective length of the separation channel was 3.6 cm.

As presented in Fig. 5, comparing the electropherograms among samples that with (a) no BSA; (b) 1×10^{-5} mg/mL BSA; (c) 7×10^{-5} mg/mL BSA; (d) 5×10^{-4} mg/mL BSA, respectively, the increment of PY peak heights at equivalent migration time was directly proportional to the amount spiked with BSA. Moreover, a linear dependence of fluorescence intensity enhancement on the concentration of BSA was obtained. To evaluate the linearity of the established method, standard curves were prepared by analyzing different concentrations of BSA between 1.00×10^{-8} and 0.10 mg/mL. The standard curves were linear in the range of 5.00×10^{-6}–1.00×10^{-3} mg/mL. The calibration equations and regression coefficient were: $Y = 622.74X + 0.03$ and $R = 0.9949$ ($n = 11$) in terms of fluorescence intensity enhancement as a function of BSA concentration. An LOD of 1.00×10^{-6} mg/mL (approx. 1.54×10^{-11} mol/L) for BSA was achieved (S/N = 3). Compared with LODs of 2.4×10^{-7} mol/L [33] and 8.00×10^{-5} mg/mL [24] in previous reports, significantly improved sensitivity and lower LODs of the protein BSA were achieved by using the label-free $EMImBF_4$ supported microchip electrophoresis with LIF detection.

3.5 Validation of the method

The experiments have also been performed with another three proteins including bovine hemoglobin, cytochrome *c*, and trypsin as samples to further validate our established method. To evaluate their linearities and LODs, standard curves were prepared by analyzing different concentrations of BSA between 1.00×10^{-8} and 0.10 mg/mL. The calibration equations and corresponding regression coefficients as well as comparisons of the results obtained between our method and previous ones have been listed in Table 1. Electropherograms of different concentrations of (A) bovine hemoglobin, (B) cytochrome *c*, (C) trypsin included in 5 μM PY–5 mM SDS–10 mM PBS are shown in Fig. 6, respectively. According to the experimentally documented findings and the comparisons of the results obtained by our method with previous ones, the feasibility and the universality of this method as well as advantages of the presented method over the existing solutions can be ensured.

4 Concluding remarks

In this study, based on the shifting dimer–monomer equilibrium of the fluorescent dye, PY, microchip electrophoresis with LIF detection technique was applied for the establishment of a label-free protein detection method. Induced by the premicellar aggregation of an anionic surfactant, SDS, PY formed nonfluorescent dimer, which largely quenches the fluorescence intensity of the PY system. However, when proteins such as BSA, bovine hemoglobin, cytochrome *c*, and trypsin were added to the solution, the formation conditions of the PY dimer were destroyed and the fluorescent monomer was recovered, then the fluorescence intensity of the system increased dramatically. The fluorescence intensity enhancement was measured by microchip electrophoresis with LIF detection in 1.2 min. When $EMImBF_4$ took the place of PBS used as supporting electrolyte, due to the excellent properties of $EMImBF_4$ and synergistic effect of $EMImBF_4$ and SDS the protein adsorption was efficiently suppressed and an about ten-fold higher fluorescence intensity was obtained than that using PBS. The linear dependence of PY fluorescence intensity enhancement on BSA concentration over the range of 5.00×10^{-6}–1.00×10^{-3} mg/mL and an LOD of 1.00×10^{-6} mg/

Table 1. Linearities and LODs of bovine hemoglobin, cytochrome *c*, trypsin used as samples, respectively and comparisons of the results obtained between our method and previous ones

Proteins	Calibration equations (our method)	Regression coefficient (our method)	Linear ranges (mg/mL)		Detection limits (mg/mL)	
			Our method	Previous method	Our method	Previous method
Bovine hemoglobin	$Y = 486.19X + 0.009$	0.9983 ($n = 10$)	5×10^{-6}–7×10^{-4}	4×10^{-4}–4×10^{-2} [34]	2×10^{-6}	2×10^{-4} [34]
Cytochrome *c*	$Y = 2645.77X + 0.008$	0.9962 ($n = 10$)	1×10^{-6}–2×10^{-4}	1.3×10^{-2}–1.3 [35]	7×10^{-7}	6.5×10^{-3} [35]
Trypsin	$Y = 3042.75X + 0.025$	0.9946 ($n = 10$)	7×10^{-7}–2×10^{-4}	0.02–0.5 [36]	5×10^{-7}	5×10^{-3} [36]

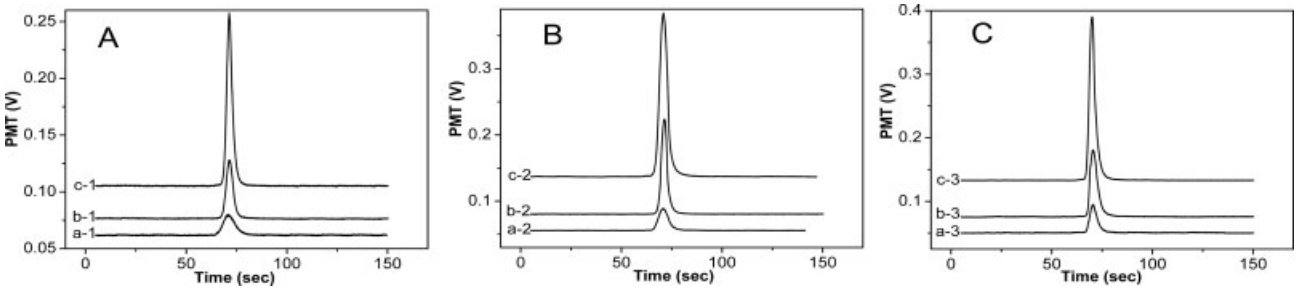

Figure 6. Electropherograms of different concentrations of (A) bovine hemoglobin, (B) cytochrome *c*, (C) trypsin included in 5 μM PY–5 mM SDS–10 mM PBS, respectively. (a-1) 1×10^{-5} mg/mL, (b-1) 7×10^{-5} mg/mL, (c-1) 3×10^{-4} mg/mL of bovine hemoglobin; (a-2) 5×10^{-6} mg/mL, (b-2) 5×10^{-5} mg/mL, (c-2) 1×10^{-4} mg/mL of cytochrome *c*; (a-3) 5×10^{-6} mg/mL, (b-3) 2×10^{-5} mg/mL, (c-3) 7×10^{-5} mg/mL of trypsin; Running buffer: 10 mM $EMImBF_4$ containing 5 mM SDS; The effective length of the separation channel was 3.6 cm.

mL confirmed the feasibility of the rapid, highly sensitive, label-free protein detection assay. With the assistance of $EMImBF_4$, tedious labeling steps relating to covalent modification of the proteins are not only avoided but also still further enhanced and expanded the inherent properties of microchip combining with the sensitivity of LIF detection technique. Surely it has great potential in expanding applications of ILs, microfluidic devices in protein detections in the future.

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The authors have declared no conflict of interest.

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