CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200700275

Ionic Liquids as Selectors for the Enhanced Detection of Proteins

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Abstract: Herein, we report an approach for protein detection enhanced by ionic liquid (IL) selectors in capillary electrophoresis (CE), with avidin as a model protein. Hydrophilic ILs were added into the running buffer of CE and acted as selectors for sample injection, enriching the positive target and excluding the negative from the capillary. When using 3% (v/v) IL selector, the detection sensitivity of avidin was improved by over one order of magnitude, while the interference from protein adsorption was effectively avoided, even in an uncoated capillary. The electrochemiluminescence method was initially used for IL-based CE with low noise that was independent of the IL concentration, making ILs almost transparent as additives in the electrophoresis buffer.

Keywords: capillary electrophoresis · electrochemiluminescence · ionic liquids · proteins · selectors

Introduction

Ionic liquids (ILs), also known as molten salts, are a new class of nonmolecular ionic solvents with low melting points (<100 °C).[1] They possess good thermal stability and ultralow volatility, which makes them "green" solvents in regard to reducing environmental contaminants.[2] Unlike conventional organic solvents, ILs possess good electrical conductivity[3] and a wide range of viscosities that are much higher than those of general solvents.[1] Furthermore, their miscibility/immiscibility with other solvents is easily controlled by the change of anions or cations. The above unique properties make ILs novel solvent systems, holding great promise for use in various fields. To date, they have widely been applied in synthesis,[4–6] catalysis,[7–9] extraction,[10–12] and separation.[13–19] In addition, a number of new applications of ILs in various fields have been reported.[20–24]

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As dialkylimidazolium-based ILs, mainly based on the 1-alkyl-3-methylimidazolium cation, were reported first,[25] this important type of IL has been wildly synthesized with specific chemical and physical properties for a variety of applications. By simply varying the length of alkyl group and/or changing the anions, the viscosities of ILs can be designed at a desired high level, allowing them to be coated onto capillaries and used as stationary phases for gas chromatography.[13–15] The intrinsic structure formed by the cation associated to an anion gives ILs a dual nature when used as additives in liquid chromatography, enhancing peak efficiencies of basic cationic solutes and changing their peak positions.[16] Similarly, when used as additives and modifiers in micellar electrokinetic chromatography, hydrophobic ILs were shown to improve the peak efficiencies and resolutions of the targets.[17] Due to their high conductivity and ionicity, aqueous solutions of ILs could be used independently as running electrolytes in capillary electrophoresis (CE).[18,19] Meanwhile, ILs could act as dynamic electroosmotic flow (EOF) modifiers, modifying the capillary wall with cation adsorption onto the wall.[18] It appears that the particular properties of ILs described could make them beneficial and promising for applications in separation methods.

Currently, there is increasing interest in the sensitive detection of proteins by using CE methods.[26–28] It is well known that a main problem of protein detection by using CE is protein adsorption onto the capillary, which may lead to a poor reproducibility or even capillary blockage. In general, it is required that an uncoated capillary is rinsed with running buffer or NaOH solution several times between runs to maintain a good performance in continuous CE

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analyses for protein detection.[26] Obviously, it is an effective method but time consuming. When dynamic modifiers, such as nonionic surfactants[29] and ILs,[19] in running electrolytes, the protein adsorption was greatly decreased. Some approaches to reducing protein adsorption by the covalent binding of modifiers onto the capillary wall were usually used as well,[30,31] giving superior efficiency and good reproducibility for protein detection. Although the above methods were good at eliminating the interference from protein adsorption, they were not beneficial to improving the detection sensitivity.

There are various methods for the enhanced detection of proteins, including the development of new sensitive detection methods,[32] labeling target proteins with highly effective probes,[33] and amplification by nanoparticles[34] or the polymerase chain reaction (PCR) technique.[26] Le et al. reported the application of an aptamer-based PCR technique for the ultrasensitive detection of proteins by using CE with laser-induced fluorescence detection. In their work, a nucleic acid aptamer was used to bind the target protein with high specificity, and then its sequence was amplified by PCR.[26] By this method, the detection of 180 protein molecules was accomplished. However, as another attractive technique, ILs have seldom been used to enhance the sensitivity of protein detection although there are a few pieces of researches on their application in protein separation.[19]

Herein, we introduce a novel use of ILs as CE selectors for the enhanced detection of proteins without adsorption interference, illustrated by the detection of avidin by using CE with electrochemiluminescence (ECL) detection. By using IL selectors, the detection sensitivity of avidin was enhanced, and the adsorption interference was greatly reduced.

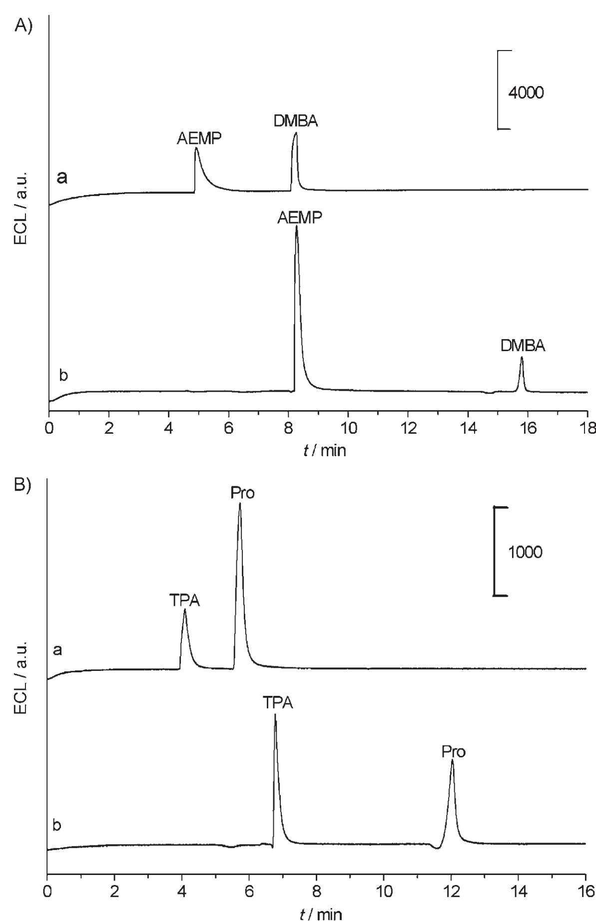

Figure 1. CE analyses with ECL detection by using 10 mM phosphate (pH 7.6) in the absence (a) and presence (b) of 1 % IL additive as running buffer. A) A mixture of 300 μM AEMP and 500 μM DMBA. B) A mixture of 150 μM TPA and 200 μM Pro. The upright bars represent the ECL intensity at 4000 a.u. (A) and 1000 a.u. (B), respectively.

Results and Discussion

To demonstrate the effect of IL additives on sample injection in the CE process, 2-(2-aminoethyl)-1-methyl-pyrrolidine (AEMP) and 4-(dimethylamino)butyric acid (DMBA) were used. In the presence of tris(2,2-bipyridyl)ruthenium(II) ($[Ru(bpy)_3]^{2+}$), AEMP and DMBA generated strong ECL emission on a platinum electrode at pH 7.6, thus the two targets could be investigated by CE-ECL. Hydrophilic 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIMBF_4$) was added to 10 mM phosphate (pH 7.6) to prepare the running buffer of CE. As shown in Figure 1A, there was an obvious increase in ECL intensity (peak height) of AEMP due to the addition of 1 % (v/v) IL to the running buffer, while that of DMBA was greatly decreased. Due to the use of IL additive, the ECL intensity of AEMP was increased to 370 %, but that of DMBA was decreased to 59 %. Although the migration times of AEMP and DMBA were obviously prolonged, the resolution of the two targets was greatly improved. Similarly, the same rules were also observed when a mixture of tripropylamine (TPA) and proline (Pro) was investigated, as shown in Figure 1B. Due to the use of 1 % IL additive, the peak intensity of TPA was increased to 217 %, while that of Pro was decreased to 51 %. Furthermore, the migration times of the targets were prolonged. In the above two cases, the use of IL additive resulted in a great increase in the electrophoresis current (data not shown). These results indicated that the $BMIMBF_4$ additive produced remarkable effects on the CE analyses.

It was noticed that, in the pH 7.6 media, AEMP and TPA were positive whereas DMBA and Pro had a negative charge. To demonstrate the close relationship between IL effect and the net charge of target molecules, Pro (the isoelectronic point is pH 6.3) was prepared in media of pH 12.3, 6.3, and 2.1, respectively. Phosphate with three pK_a values (12.3, 7.2, 2.1) was chosen as the sample buffer because this buffer could hold the pH of samples stable at the above values. In the three cases, Pro was negative, neutral, or with little positive charge, and its ECL intensity was obviously affected by the IL additive to different extents (Figure 2). Due to the use of 1 % IL additive, there was a corresponding decrease of 32, 54, or 69 % in ECL intensity of Pro, indi-

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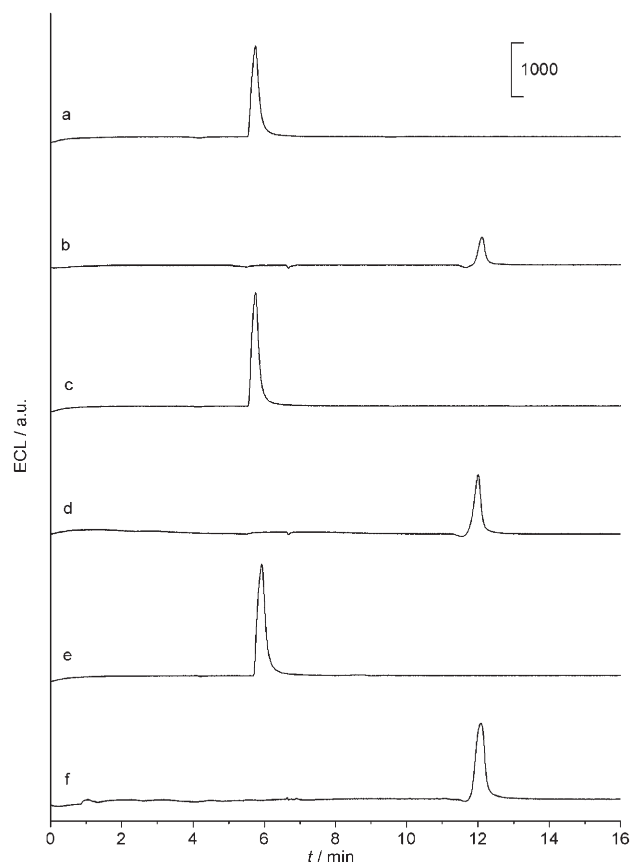

Figure 2. CE-ECL analyses for Pro dissolved in phosphate buffer solutions at different pH values by using 10 mM phosphate in the absence (a, c, and e) and presence (b, d, and f) of 1% IL additive as the running buffer. a,b) 300 μM Pro dissolved in pH 12.3 phosphate, c,d) 300 μM Pro dissolved in pH 6.3 phosphate, e,f) 300 μM Pro dissolved in pH 2.1 phosphate. The upright bar represents the ECL intensity at 1000 a.u.

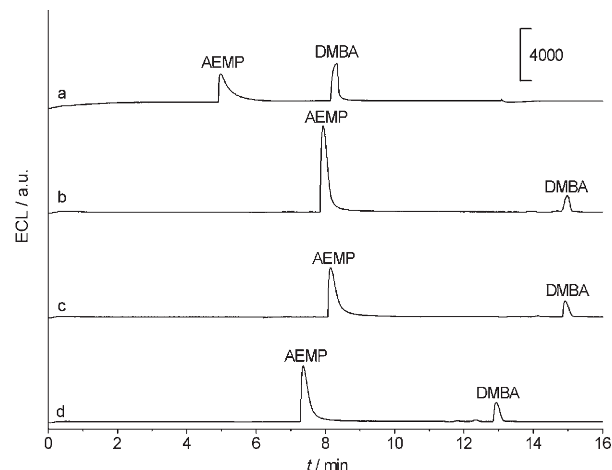

Figure 3. CE-ECL analyses for a mixture of 300 μM AEMP and 500 μM DMBA by using 10 mM phosphate in the absence (a) and presence (b, c, and d) of IL additives as running buffer: b) 53.5 mM $EMIMBF_4$, c) 53.5 mM BMIMCl, d) 53.5 mM EMIMCl. The upright bar represents the ECL intensity at 4000 a.u.

cating that the decrease was directly dependent on the negative net charge of the target when the other conditions were unchanged. And also, under the same conditions, the increase of the AEMP peak was more remarkable than that of TPA (Figure 1). There were two amino groups existing in AEMP, whereas there was only one in TPA, thus AEMP had more positive net charge than TPA in pH 7.6 phosphate. Accordingly, a general rule for IL effect on the CE analyses of various targets could be summarized: The IL additives enhanced greatly the peak intensity of positive targets, whilst that of those with negative, no, or little positive net charge was obviously decreased.

To test the generality of the IL effect, other hydrophilic 1-alkyl-3-methylimidazolium-based ILs, such as 1-ethyl-3-methylimidazolium tetrafluoroborate ($EMIMBF_4$), 1-butyl-3-methylimidazolium chloride (BMIMCl), and 1-ethyl-3-methylimidazolium chloride (EMIMCl) were also used as additives when AEMP and DMBA were investigated by CE-ECL (Figure 3). Similar to $BMIMBF_4$, the other three IL additives led to an increase in ECL intensity of AEMP, while that of DMBA was decreased. The prolonged migration times and improved resolution of the two targets were observed as well. This indicated that the effect of IL additives existed throughout all the experiments, independent of the anions of the ILs or the length of their alkyl groups. But the four ILs used affected the signal intensities and migration times of targets to different extents when they were used at the same concentration (the molar concentration of 1% $BMIMBF_4$ is 53.5 mM). Among these ILs, $BMIMBF_4$ had a maximal effect on the detection of AEMP and DMBA, while EMIMCl had a minimal one. It was suggested that the effect of 1-alkyl-3-methylimidazolium-based ILs was mainly dependent on their molecular properties, such as the hydrophilicity of anions and the length of alkyl groups. Accordingly, $BMIMBF_4$ was chosen for the following experiments. Hydrophobic IL additives were not tested here.

To reveal the relationship between IL effect and its concentration, AEMP and DMBA were investigated by CE-ECL with $BMIMBF_4$ of various concentrations as the additive in running buffer. As shown in Figure 4A, the increase of AEMP and decrease of DMBA was directly proportional to the IL concentration, indicating that the IL effect was easily controlled by its concentration. When 4% of the IL additive was used, the ECL intensity of AEMP increased to 1330%, whilst the peak of DMBA was hardly observed, which meant that DMBA was not allowed by the IL additive to inject into the capillary. In addition, no noticeable changes in the background noise were observed throughout, as shown in Figure 4B. This meant that the use of IL additive, even at a high concentration, had little effect on the background of the CE-ECL analysis, which was significant for the application of the ECL method to IL-based analysis. In general, most of the ILs contained one or more aromatic group, making these substances not transparent for ultraviolet or fluorescent detection. The use of IL additives in the electrophoresis electrolyte would attribute significantly to the background signal, which limited somewhat the utility of

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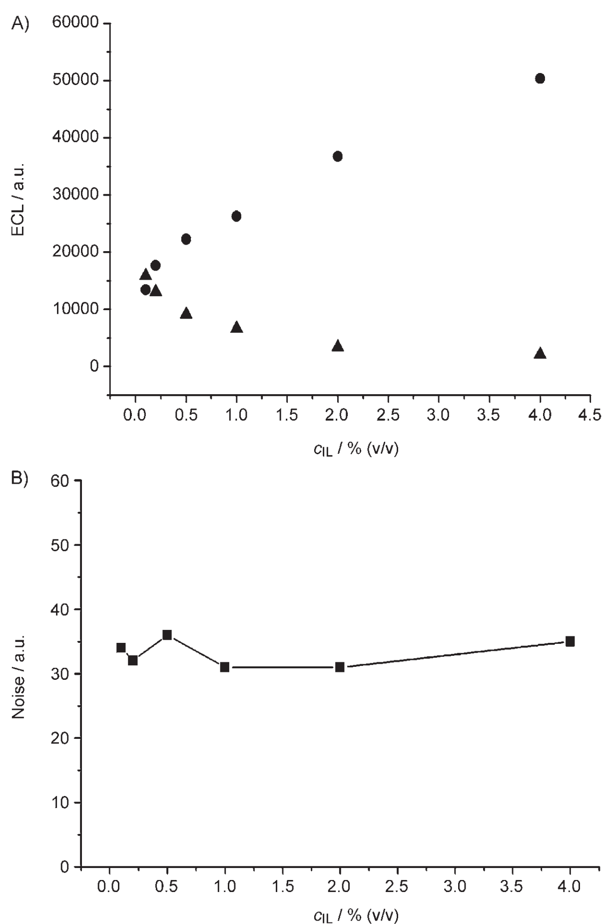
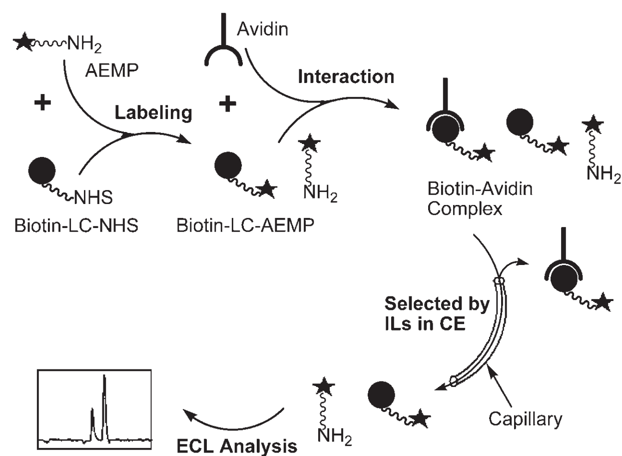


Figure 4. Effect of IL additive on the ECL intensity of targets and the background signal. A) Dependence of ECL intensities (peak area) of 300 μM AEMP (●) and 500 μM DMBA (▲) on the IL concentration. B) Dependence of the average noise on the IL concentration.

ILs. However, the $[Ru(bpy)_3]^{2+}$-based ECL detection was hardly influenced by the existence of ILs. When the IL additive of 4% $BMIMBF_4$ (214 mM) was used, the noise was almost not increased (Figure 4B). So the ratio of signal to noise (S/N) for the detection of AEMP and DMBA was dependent on the concentration of IL additive. For AEMP detection, the S/N ratio was improved by over one order of magnitude when $BMIMBF_4$ was increased to 4%, whereas that of DMBA was close to zero. Obviously, the injection capacity of the targets could be controlled by the IL concentration. By the use of IL additive of an appropriate concentration, only the positive of target of analytes was allowed to enter the capillary. Accordingly, selective sample injection in the CE process was accomplished.

The selectivity of an IL in CE might be attributed to its unique properties. The good conductivity of ILs enhanced that of running buffer when the additive was used, making the resistance of sample solution much higher than that of running buffer. Thus, under the same conditions, the actual voltage for sample injection was greatly increased compared with that when no IL additive was used, which meant that the field-amplified sample stacking effect was accomplished. It is well known that this effect makes analytes, especially the positive analytes, greatly enriched at the electrokinetic injection mode. On the other hand, the addition of ILs into running buffer led to a decreased EOF, which accounted for the prolonged migration times of target analytes. The decreased EOF brought a negative effect on the injection capacity of any analyte and reduced it to a great extent. For analytes with negative, no, or little positive net charge, such as DMBA and Pro, the field-amplified effect was not strong and the negative effect of the IL was dominant, resulting in a decrease in the capacity of electrokinetic injection. For those with a much more positive charge (AEMP and TPA), however, the field-amplified effect of the IL was much stronger than the negative effect, thus the samples were greatly enriched.

As we all knew, the detection sensitivity of targets could be greatly improved by preparing samples in a low-conductivity solution (for example, pure water).[35] It should be noted that this sample stacking technique enhanced the detection of both the positive and negative targets, as reported previously.[36] However, the IL additives served as novel CE selectors here, enhancing the positive and excluding the negative from the capillary. This selectivity of ILs was of great significance for CE-based bioanalysis (for example, protein detection), which was illustrated by the detection of avidin by using the CE-ECL method (Scheme 1). Based on the specific and quantitative combination of avidin to biotin or its derivatives, the target protein could be indirectly detected by measuring the decrease of biotin. A derivation method with an ECL probe described in our previous work[33] was used here. Succinimidyl-6-(biotinamido)hexanoate (biotin-LC-NHS) was labeled with AEMP to produce biotin-LC-AEMP with high ECL activity. This biotin derivative bound avidin with high affinity to form a macromolecule complex, resulting in an obvious decrease in the ECL signal of biotin-LC-AEMP. The whole process of labeling and detection was monitored by CE-ECL, as shown in Figure 5.



Scheme 1. Schematic of avidin detection without adsorption interference by using CE-ECL analysis assisted by IL selectors.

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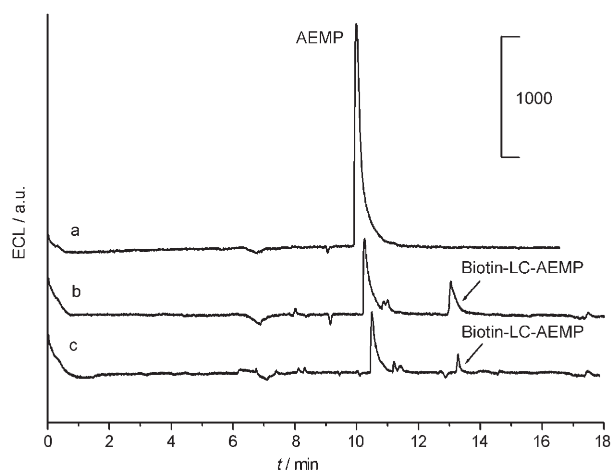

Figure 5. Avidin detection by using CE-ECL analysis assisted by 3% IL selector. a) 1.5 μM AEMP, b) after the addition of 1 μM biotin-LC-NHS into solution a, c) after the addition of 5×10^{-6} g mL^{-1} avidin into solution b. The upright bar represents the ECL intensity of 1000 a.u.

Due to the selectivity of the IL, the additive made the injection capacity of biotin-LC-AEMP increase greatly. Similar to AEMP, this biotin derivative had a much more positive charge in neutral media. So the IL selector at a high concentration had a strong enhancing effect on the detection of biotin-LC-AEMP. As shown in Figure 6, its ECL intensity was improved more than 10 times by the use of 3% $BMIMBF_4$, resulting in an improved detection sensitivity of avidin. Under the conditions, a detection limit of 12.5 nM (or 40 amol, the injection volume is 3.2 nL) for avidin was obtained (see the Supporting Information), which was improved by over one order of magnitude compared with that reported previously.[33] This sensitive method made it possible to perform avidin detection at a much lower concentration (less than 10%) compared to the use of no IL additive,

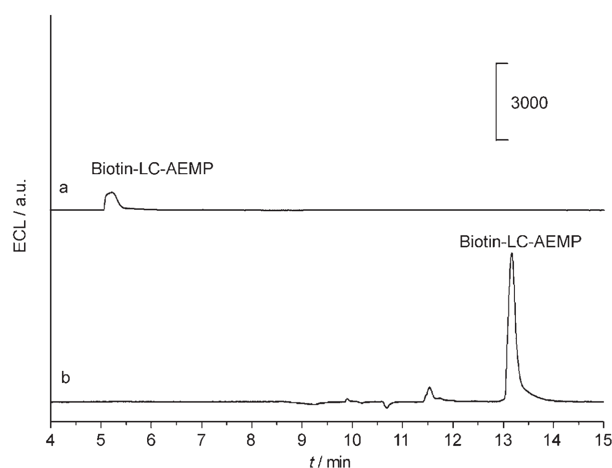

Figure 6. Detection of 200 μM biotin-LC-AEMP by CE-ECL with 10 mM phosphate (pH 7.6) in the absence (a) and presence (b) of 3% IL selector as running buffer. The upright bar represents the ECL intensity of 3000 a.u.

which was beneficial to reducing protein adsorption. On the other hand, the protein complex (avidin/biotin) was excluded from the capillary by the use of 3% IL additive. Similar to Pro, the protein complex was an amphoteric molecule with an isoelectric point of pH 10–10.5, and it had little positive net charge in a phosphate buffer of pH 7.6. But its charge/mass ratio was close to zero due to a high molecular weight. When the IL additive was used at 3%, the protein complex injected into the capillary was drecreased to about 10%. Accordingly, protein adsorption onto the capillary was reduced to less than 1% compared to when the IL selector was not used. Under these conditions, a good reproducibility for repetitive detection of avidin was obtained (see the Supporting Information), indicating that there was little adsorption interference because the protein complex was excluded from the capillary. Theoretically, the IL additives of a higher concentration were more beneficial to avidin detection, but it was at the cost of the analysis time. So it was preferable that the selectors were used at an appropriate concentration, which accounted for the 3% $BMIMBF_4$ adopted in our experiments.

Conclusion

In this work, ILs have been used as novel CE selectors for the enhanced detection of proteins without adsorption interference. Hydrophilic ILs were added into the running buffer and acted as the selectors for sample injection in the CE process, enriching positive targets and excluding the negative from the capillary. The selectivity of ILs were of great significance for protein detection, which was illustrated by the detection of avidin by using CE-ECL analysis. Here the ECL method was for the first time applied to IL-based CE with low noise that was independent of the IL concentration, making ILs almost transparent as additives in CE. By the use of 3% $BMIMBF_4$ additive, the detection sensitivity of avidin was improved by over one order of magnitude, while the interference from protein adsorption was almost avoided. It was suggested that the IL selectors held great promise for application in protein detection. By the use of this technique, plenty of proteins, such as streptavidin and other immunoproteins, could be sensitively detected without adsorption interference when appropriate detection methods were adopted.

Experimental Section

Materials: All ILs were obtained from Fluka Chemie (Buchs, Neu-Ulm, Switzerland). AEMP, DMBA, TPA, Pro, and $[Ru(bpy)_3]^{2+}$ were purchased from Sigma–Aldrich (St. Louis, MO, USA). Biotin-LC-NHS and immunopure avidin were obtained from Pierce (Rockford, IL, USA). A 50 mM $[Ru(bpy)_3]^{2+}$ stock solution dissolved in water was stored at 4°C in a refrigerator. The 10 mM working solution was freshly prepared by diluting the stock solution with 0.1 M phosphate (pH 7.6) just before use. Water (18 MΩ) purified by a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

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Instrumentation: A CE-ECL setup described in our previous work[37] was used in this work. Briefly, an uncoated fused-silica capillary (Yongnian Optical Fiber, Hebei, China) with 25 μm inner diameter was cut to 60 cm in length and used for CE separation. This was rinsed with 0.1 M NaOH to clean the inner wall when not used. A Model CHI800 Voltammetric Analyzer (CH Instruments, Austin, TX) was used for electrochemical measurements in ECL experiments. A high-voltage power supply (Shanghai Nucleus Institute, Shanghai, China) was used to supply high voltage for CE. Samples were injected at 20 kV for 10 s in the electrokinetic mode. Electrophoresis was performed at ambient temperature with a voltage of 20 kV by using 10 mM phosphate (pH 7.6) in the presence or absence of IL additives as running buffer. The ECL emission was detected on a platinum disk electrode of 500 μm diameter at 1.2 V (versus Ag/AgCl) with a Model MCDR-A Chemiluminescence Analyzer Systems (Xi'An Remax Science & Technology, Xi'An, China). The distance between the working electrode and the end of the capillary was 125 μm. The voltage of photomultiplier tube was set at 850 V.

ILs used: Four hydrophilic 1-alkyl-3-methylimidazolium-based ILs, $BMIMBF_4$, $EMIMBF_4$, BMIMCl, and EMIMCl, were used in our experiments. These ILs were added to 10 mM phosphate (pH 7.6), which was used as the running buffer of CE. As the control experiments, the phosphate buffer containing no additive was also used as the electrophoresis buffer.

ECL labeling and avidin detection: In our experiments, labeling of biotin-LC-NHS with AEMP was performed in phosphate buffer to produce biotin-LC-AEMP with high ECL activity. Typically, biotin-LC-NHS (0.45 mg) was dissolved in dimethylformamide (10 μL) and then phosphate buffer (840 μL, 0.1 M, pH 7.6) was added, followed by AEMP (150 μL, 10 mM). The mixture was shaken for 20 min and continued to stay at room temperature for 2 h, and was then diluted with water to 10 mL followed by dilution with phosphate buffer to various working concentrations. Avidin was indirectly detected by CE-ECL, based on the measurement of biotin-LC-AEMP. The reaction between biotin and avidin was performed in diluted phosphate for 1 h at ambient temperature. The detection limit of avidin was obtained by adding the protein into a solution of 100 nM biotin-LC-AEMP (see the Supporting Information).

Acknowledgements

This work is supported by the National Natural Science Foundation of China with the grants 20335040, 20427003, and 20675078 and the Chinese Academy of Sciences, grant KJCX2.YW.HO9.

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Received: February 16, 2007
Revised: May 18, 2007
Published online: July 18, 2007

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