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Short communication

Use of disposable open tubular ion exchange pre-columns for in-line clean-up of serum and plasma samples prior to capillary electrophoretic analysis of inorganic cations

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ABSTRACT

A simple analytical system using disposable, open-tubular ion exchange clean-up precolumns coupled in-line to capillary electrophoresis for direct injection of biological samples is presented. The clean-up precolumns were prepared from fused silica capillaries by thermally initiated layer-by-layer polymerization of poly(butadiene-maleic acid) (PBMA) directly on the capillary wall. Typically, 6 cm long precolumns with 4-layers of PBMA were used for sample pretreatment. A robust and reproducible coupling between the precolumn (75 μm ID) and the analytical capillary (50 μm ID) was achieved using an inexpensive, commercially available low dead volume union. No extra dispersion of the analyte zones was observed. Proteins and other high molecular weight compounds from biological sample matrices were retained on the cation-exchanger sites of the precolumn, which eliminated their adsorption on analytical capillary walls and ensured stable electroosmotic flow and migration times of target analytes. Unretained small inorganic cations migrated freely into the analytical capillary for separation and detection. Applicability of the sample clean-up procedure was proved by determination of major inorganic cations in blood serum and plasma samples using capillary electrophoresis with contactless conductivity detection. Separations were performed in background electrolyte solution consisting of 15 mM L-arginine, 12.5 mM maleic acid, 3 mM 18-crown-6 at pH 5.5 and repeatabilities of migration times and peak areas were below 1.5% and 7.3%, respectively. Less than 1 μL of biological sample was required for injection.

1. Introduction

Analysis of biological samples has its own difficulties, because of the usually limited sample amount, the low analyte concentrations and the complex sample matrices. Capillary electrophoresis (CE) can successfully handle the low sample volumes and in some instances even the low concentrations, however, the influence of matrix compounds on CE separation performance presents a major limitation for this type of samples [1]. The sample matrix and presence of various biologically active species (often charged and in different chemical forms) interfere significantly with the instrument and its components, most notably with performance of fused silica (FS) separation capillaries. Matrix components adhere to the capillary surface, leading to various effects such as changes of electroosmotic flow (EOF), peak-broadening due to analyte binding to the surface-adsorbed proteins and detection interferences [2,3].

Direct injection of biological samples in CE is thus complicated and sample pretreatment is usually required. Traditional sample pretreatments such as liquid–liquid extraction [4], solid-phase extraction/microextraction [5] or precipitation followed by centrifugation [6] can be used to remove proteins. These procedures are often time consuming, costly, require large sample volumes and necessitate use of organic solvents and additional instrumentation. Usually, they cannot be combined on-line with CE. Alternative approaches for clean-up of biological samples include ultrafiltration [7,8], electrodialysis [9,10], liquid phase microextraction [11–13] and electric field driven sample pretreatment [14–16]. Note however, that even though these techniques offer higher selectivity, lower the consumption of organic solvents and reduce the pretreatment time, they are mostly performed off-line and additional sample handling is therefore required.

In this contribution a simple concept for clean-up of biological samples is presented using short disposable, open-tubular (OT) precolumn coupled in-line to CE capillary. Interfering compounds

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from the injection plug are adsorbed onto the surface of the precolumn and stable CE performance is achieved. Moreover, this system allows for easy handling the capillaries, such as flushing with background electrolyte (BGE) and injecting samples. The coupling is sufficiently robust and simple to construct with a commercially available low dead volume union and induces no additional sample dispersion. The precolumn is easily connected/disconnected to/from CE capillary and can be simply disposed off after each use. Performance characteristics of the proposed system are demonstrated on analysis of small inorganic cations in blood serum and plasma samples.

2. Materials and methods

2.1. Instrumentation

2.1.1. Electrophoretic system

A purpose-built CE instrument was employed for all electrophoretic runs. A high voltage power supply unit (Spellman CZE2000R, Start Spellman, Pulborough, UK) was operated at +15 kV (at the injection side). The analytical capillary (FS, 50 μm ID, 375 μm OD, L_{tot} 47 cm, L_{eff} 40 cm, Polymicro Technologies, Phoenix, AZ, USA) was preconditioned for 10 min each with 1 M NaOH, deionized (DI) water and BGE solution. Between two successive runs, the capillary was flushed with BGE solution for 2 min. Injection was carried out hydrodynamically by elevating the sample vial to a height of 30 cm for 30 s representing 30 nL injection volume. All CE experiments were performed at ambient temperature.

2.1.2. Detection system

A capacitively coupled contactless conductivity detector (C^4D) described recently by [17] was used. It consists of a detector cell, an external ac voltage source for excitation and an external detector circuitry for processing the cell current. The detector was operated at 120 kHz and 300 V_{pp} in all experiments. Data were collected using a Panther-1000 (Ecom, Praha, Czech Republic) data acquisition system.

2.2. Preparation of OT sample clean-up precolumns

Preparation of poly(butadiene-maleic acid) (PBMA) based OT columns was described previously [18]. A thin layer of PBMA prepolymer mixed with 5% azobisisobutyronitrile (AIBN) was coated onto a 1 m long FS capillary (75 μm ID, 375 μm OD, Microquartz GmbH, Munich, Germany) and crosslinked at 160 °C for 15 min. The coating procedure was repeated *k*-times to create a *k*-layered precolumn. The prepared column was eventually cut into pieces of various lengths (3–12 cm). These precolumns were connected to the analytical capillary through a low dead volume union (P-772, Upchurch Scientific, Oak Harbor, WA, USA). A sketch of the connection between the OT sample clean-up precolumn and the analytical capillary is shown in Fig. 1.

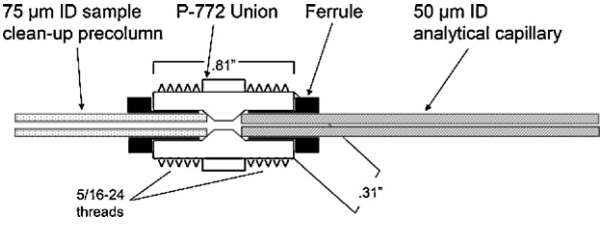

Fig. 1. Interfacing of the clean-up precolumn and the analytical capillary using the low dead volume union.

2.3. Reagents, electrolytes, standards, and samples

All chemicals were of reagent grade and DI water was used throughout. All multi-ion standard solutions were prepared daily from stock solutions of inorganic cations (250 mM) and were diluted with DI water; chloride and sulphate salts were purchased from Pliva-Lachema, Brno, Czech Republic. For precolumn preparation, PBMA prepolymer (42% solids in aq. solution, Polysciences, Eppelheim, Germany) and AIBN (Acros, Geel, Belgium) were used. Human serum albumin (HSA) was purchased from Sigma, Steinheim, Germany. BGE solutions for CE measurements were prepared daily from stock solutions of L-arginine (100 mM), maleic acid (100 mM) and 18-crown-6 (250 mM); chemicals were purchased from Sigma. Samples of human serum and plasma (Sigma) were purchased as lyophilized powder and were prepared according to supplier's instructions. 1 μL of the sample was diluted with 79 μL of DI water and 20 μL was pipetted into a plastic microvial for injection; all biological samples were stored in deep freezer at less than −20 °C.

3. Results and discussion

3.1. Effect of the column connection on CE performance

The BGE solution used for determination of inorganic cations in biological fluids was optimized previously [19] and consisted of 15 mM L-arginine, 12.5 mM maleic acid and 3 mM 18-crown-6 at pH 5.5. This BGE solution was used to evaluate robustness and performance of the connection between the precolumn and the analytical capillary.

First, a standard solution of K^+, Na^+, Ca^{2+} and Mg^{2+} at concentration levels corresponding to human serum diluted 1:80 was separated in a 47 cm long analytical capillary. Then, 6 cm of the same analytical capillary was cut off and the two sections (6 and 41 cm) were re-connected using a P-772 union. Finally, a 4-layer PBMA sample clean-up precolumn (6 cm) was connected to the 41 cm analytical capillary through the P-772 union. Seven measurements of the standard solution were repeated in each of the above described separation system and several parameters were evaluated as summarized in Table 1. Fig. 2 shows separation of the four cations in the three separation systems – no deterioration of the CE performance in terms of separation efficiency and sensitivity was observed for the capillaries connected through the union (Fig. 2b and c) compared to a single-piece analytical capillary (Fig. 2a). Repeatability of migration times and peak areas was similar for all three separation systems (RSD values 0.1–0.2% and 0.6–5.0%, respectively) with no effect on the number of theoretical plates. A slightly better resolution was obtained for Na^+/Ca^{2+} and Ca^{2+}/Mg^{2+} analyte pairs when PBMA precolumn was connected to the analytical capillary. This was due to interaction of Ca^{2+} and Mg^{2+} ions with the ion-exchanger sites of the precolumn [20], and resulted in their prolonged migration times. However, no deteriorating effect on separation efficiency of the two cations was observed, as the N values were slightly better with the PBMA precolumn.

To evaluate the reproducibility of the connection using the P-772 union, three different 4-layer PBMA precolumns were cut (6 cm each) and were connected/disconnected/reconnected five times to the analytical capillary. Complete precolumn replacement procedure in our home-made CE system took less than 1 min. PBMA precolumns can also be connected to analytical capillaries in commercial CE cartridges (e.g. Agilent), the only difference is the precolumn length (10 cm) and replacement time (2–3 min). Migration times and peak areas were measured for the standard solution of the four cations and repeatability results are summarized in Table 1. The RSD values were better than 5.5% for peak

areas and are similar to the values for a single clean-up precolumn connected to the analytical capillary as also presented in Table 1.

A slight deterioration of the repeatability of migration times was observed, which might be remedied by including an internal standard (such as Li^+) if necessary. However, as the composition of biological samples is quite constant and only the four cations migrate in this time window of the CE measurements, identification of all peaks is straightforward and internal standard was not necessary in our measurements.

3.2. Effect of ion exchange capacity and length of PBMA precolumns on sample clean-up

In biological samples, proteins adsorb on the separation capillary walls and cause increase in migration times of the analytes for each successive injection, rendering the quantitation difficult. The amount of proteins that can be removed by the precolumn depends on its ion exchange capacity, which is in turn dictated by the number of PBMA layers deposited. Too high ion exchange capacity may, however, have a detrimental effect on the separation of cationic analytes because they also interact with the cation exchanger sites. To optimize the precolumn composition and length, five precolumns with 2, 4, 6, 8 and 10 PBMA layers were prepared, connected to the analytical capillary and their effect on sample clean-up and CE separation was tested. Their length was varied between 3 and 12 cm. The standard solution of four inorganic cations with addition of 1 g/L HSA as a model protein was injected five-times into the separation system with each precolumn attached. The injection volume was ca. 30 nL, see Section 2.1. The column breakthrough capacity (expressed as number of injections) was estimated based on the stability of migration times of inorganic cations [10]. As expected, the capacity was lowest for all three lengths of 2-layer PBMA precolumns. Unstable CE performance was observed already after the second (3 and 6 cm precolumn) and the third (12 cm precolumn) injection due to adsorption of the unretained HSA onto the analytical capillary walls. Precolumns with 4 or more layers of PBMA (6 and 12 cm) showed a stable performance for at least 5 successive injections. On the other hand, in columns with 8 and 10 PBMA layers a strong interaction of Ca^{2+} and Mg^{2+} with the precolumn was observed resulting in longer migration times and peak shape deterioration. Even with shortest precolumn (3 cm) their peak shapes did not improve.

A 4-layer PBMA precolumn of 6 cm was therefore used in all subsequent experiments due to its good capacity to retain proteins such as HSA and due to the faster preparation compared to precolumns with more PBMA layers. The precolumn can be used for at least five repetitive measurements of solutions with high protein concentration but can also be used as a single-use extraction unit, when needed. If higher number of clean-ups per precolumn is required, longer precolumns or more PBMA layers (maximum 6 layers) may be applied.

3.3. Direct injection of blood serum and plasma samples

Blood serum and plasma were diluted 1:80 with DI water and were first injected into the analytical capillary without pretreatment precolumn. Separation of all inorganic cations was achieved in both samples. In Fig. 3, the constant increase in migration times can be clearly observed for 15 consecutive injections of blood serum (full symbols) because the proteins from serum gradually adsorbed to the inner walls of the analytical capillary [10]. A 4-layer PBMA sample clean-up precolumn (6 cm) was then in-line coupled to the analytical capillary for clean-up of these samples. Because the PBMA precolumns have a limited capacity and a single precolumn does not allow for 15 repeated sample clean-ups, in total three precolumns were used for each sample. Five direct injections of

Table 1
Analytical parameters for analysis of the standard solution of four inorganic cations in a 47 cm long analytical capillary, and a 41 cm long analytical capillary connected to 6 cm FS capillary and to 6 cm 4-layer PBMA precolumn, $n = 5$; and effect of connecting three various 4-layer PBMA precolumns to analytical capillary on repeatability of CE–C^4D of biological samples, $n = 15$. Standard solution and CE conditions are as for Fig. 2.

	K^+	Na^+	Ca^{2+}	Mg^{2+}
Peak area (RSD, %), $n = 5$				
AC	2.8	2.4	1.7	1.6
FS + AC	3.0	0.6	5.0	2.1
PBMA + AC	2.9	1.1	2.9	1.8
Migration time (RSD, %), $n = 5$				
AC	0.1	0.1	0.2	0.2
FS + AC	0.2	0.2	0.2	0.2
PBMA + AC	0.2	0.2	0.2	0.2
Average N (theoretical plates/m), $n = 5$				
AC	49,000	7100	114,000	141,000
FS+AC	49,500	7200	113,000	147,000
PBMA+AC	50,500	7200	115,000	158,000

	K^+/Na^+	Na^+/Ca^{2+}	Ca^{2+}/Mg^{2+}
Average resolution (R_s), $n = 5$			
AC	3.6	0.8	1.7
FS + AC	3.6	0.9	1.8
PBMA + AC	3.6	1.0	1.9

	K^+	Na^+	Ca^{2+}	Mg^{2+}
Peak area, (RSD, %), $n = 15$				
Standard	3.7	5.5	3.2	2.7
Human serum	5.9	4.5	3.7	3.7
Human plasma	7.3	2.3	2.2	7.2
Migration time (RSD, %), $n = 15$				
Standard	1.5	1.2	1.2	1.0
Human serum	1.4	1.2	1.3	1.2
Human plasma	0.7	1.0	1.0	1.1

AC – 47 cm FS analytical capillary only.
FS + AC – 6 cm FS capillary + 41 cm FS analytical capillary.
PBMA + AC – 6 cm 4-layer PBMA capillary + 41 cm FS analytical capillary.
Serum and plasma samples were diluted 1:80 with DI water.

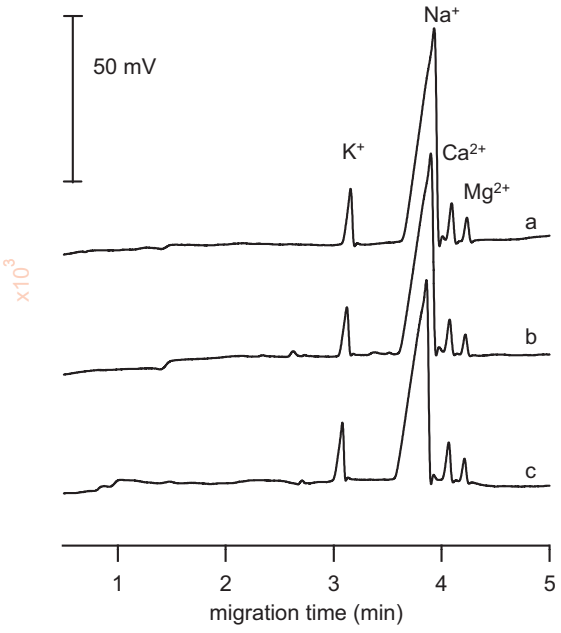

Fig. 2. Determination of inorganic cations in standard solution (0.075 mM K^+, 1.8 mM Na^+, 0.04 mM Ca^{2+}, 0.025 mM Mg^{2+}) using, (a) 47 cm FS analytical capillary, (b) 6 cm FS + 41 cm FS analytical capillary, and (c) 6 cm 4-layer PMBA precolumn + 41 cm FS analytical capillary. CE conditions: BGE solution, 15 mM L-arginine, 12.5 mM maleic acid, 3 mM 18-crown-6, pH 5.5; hydrodynamic injection from 30 cm for 30 s, separation voltage +15 kV.

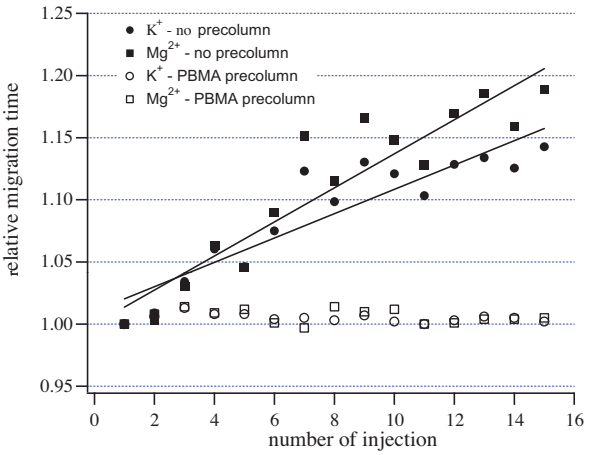

Fig. 3. Effect of precolumn on migration times for direct injection of a serum sample (diluted 1:80 with DI water). 15 consecutive injections were performed without (full symbols) and with (open symbols) a 6 cm 4-layer PBMA precolumn. CE conditions are as for Fig. 2a and c.

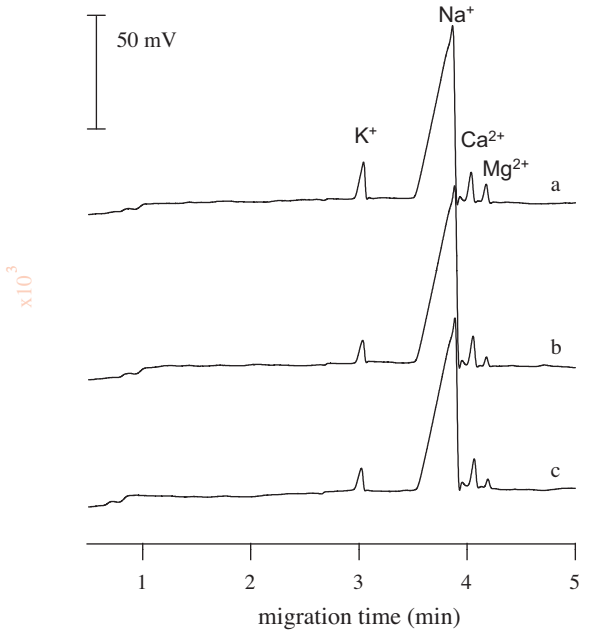

Fig. 4. Direct injection of biological samples using sample clean-up precolumn, 6 cm 4-layer PBMA, in-line coupled to 41 cm FS analytical capillary; (a) standard solution, (b) serum, (c) plasma, concentrations in standard solution and CE conditions are as for Fig. 2c, serum and plasma dilution 1:80 with DI water.

blood serum and blood plasma were performed with each precolumn. Excellent stability of migration times for both samples was achieved, as shown for blood serum in Fig. 3 (open symbols).

Repeatability data for CE–C^4D analyses of the cleaned-up samples are summarized in Table 1 and electropherograms for direct injection of the standard solution, blood serum and blood plasma are shown in Fig. 4. The performance of the sample clean-up precolumn coupled in-line to CE was very good, showing that positively charged proteins and other interfering compounds from biological samples are retained by the cation exchanger in the precolumn. The sample matrix contains also anionic and neutral compounds but as the EOF is reduced in the BGE solution (1.9×10^{-8} m^2/Vs), most anionic compounds migrate in opposite direction and do not enter the separation capillary. Neutral and slow anionic matrix compounds are dragged by the EOF through the precolumn and potentionally may penetrate into the separation capillary. Since no deterioration of CE performance was observed, we assume that under the CE conditions employed, these species are either eliminated by adsorption onto the precolumn or they do not adsorb onto the capillary wall and thus have negligible effect on CE performance.

4. Conclusions

OT ion exchange precolumns were for the first time used for sample clean-up and direct injection of biological samples in CE. Preparation of these precolumns is simple, fast and inexpensive. They can therefore be used as disposable sample pretreatment units which can be simply discarded after each use. A low dead volume union provides a robust and reproducible connection between the precolumn and the analytical capillary, with no deterioration of CE performance. It has been demonstrated that cation exchanger moieties in the precolumn effectively retain positively charged proteins and other high MW compounds from the sample matrix, whereas small inorganic cations migrate freely into the separation capillary. Using the described sample clean-up precolumns eliminates necessity for laborious off-line sample pretreatment or frequent capillary equilibration with NaOH. The developed sample pretreatment method was demonstrated on determination of small inorganic cations in blood serum and plasma samples. As there exist various chemistries of the deposited layers, the OT approach can be applied for removal of compounds having different properties, for instance use of an anionic or hydrophobic coating could be used to remove anionic and non-polar interferents, respectively. Work is in progress to further evaluate these possibilities on direct injection and analysis of other analytes in various complex matrices.

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