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Design and evaluation of a coupled monolithic preconcentrator-capillary zone electrophoresis system for the extraction of immunoglobulin G from human serum

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Abstract

The analysis of proteins in biological fluids by capillary electrophoresis (CE) is of interest in clinical chemistry. However, due to low analyte concentrations and poor concentration limits of detection (CLOD), protein analysis by this technique is frequently challenging. Coupling preconcentration techniques with CE greatly improves the CLOD. An on-line preconcentration-CE method that can selectively preconcentrate any protein for which an antibody is available would be very useful for the analysis of low abundance proteins and would establish CE as a major tool in biomarker discovery. To accomplish this, the development of an on-line protein G monolithic preconcentrator-CE device is proposed. To generate active groups for protein immobilization, glycidyl methacrylate (GMA) was used to prepare polymer monoliths. A 1.5–2 cm monolith was cast inside a 75 μm I.D. fused silica capillary that had previously been coated with alternating layers of negatively (dextran) and positively (polybrene) charged polymers. Protein G was covalently bound to GMA. Monoliths from different formulations were prepared and evaluated for binding capacity to optimize the monolith formulation for protein preconcentration. The physical properties of the column considered best for preconcentration were determined by mercury intrusion porosimetry. The total pore area was 4.8 m^2/g, the average pore diameter was 3.3 μm and the porosity was 82%. The monolith had a low flow resistance and was macroscopically homogeneous. The effectiveness of the monolith to rapidly preconcentrate proteins at flow rates as high as 10 μL/min was demonstrated using a 1.8 μM IgG solution. This system proved effective for on-line sample extraction, clean-up, preconcentration, and CE of IgG in human serum. IgG from diluted (500 and 65,000 times) human serum samples was successfully analyzed using this system. The approach can be applied to the on-line preconcentration and analysis of any protein for which an antibody is available.

Keywords: Proteins; Preconcentration; Monolith; Protein G; Capillary electrophoresis; IgG; Glycidyl methacrylate; Human serum

1. Introduction

Application of capillary electrophoresis (CE) in proteomics research continues to gain popularity [1]. However, in order to make CE more attractive to real bioanalytical applications, some drawbacks still need to be addressed. One of the most striking drawbacks is the restriction in sample volume that can be injected into the capillary to preserve high column efficiency. This coupled with the short path length for optical detection leads to poor concentration limits of detection [2]. For proteins, CE analysis is usually limited to the micromolar range when using UV absorption detection [3]. To compensate for this, different capillary geometries, novel optical designs, and sample preconcentration methods have been developed [2].

An approach to circumvent poor concentration detection limits in CE is to use a more sensitive detector, such as laser induced fluorescence (LIF), electrochemical or mass spectrometry (MS). Another approach is to increase the sample loadability by using techniques such as field-amplified stacking and transient isotachophoresis [4,5].

The typical approach to analyze components at low concentrations in complex matrices is to preconcentrate the analytes either on-line or off-line prior to separation. Even though they are more flexible, off-line preconcentration methods have the disadvantage that sample handing may lead to analyte losses on exposed surfaces (e.g., vials, tips, and pipets) [6]. Minimal

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sample handing can be achieved by the use of on-line preconcentration methods [5,7].

Much effort has been directed toward the development of on-line sample preconcentration in CE, and several papers can be found dealing with the preconcentration of trace components present in human specimens prior to separation [8–13]. Several on-line preconcentration systems for CE have been designed, in which a solid support (e.g., polymeric or silica based particles) is either positioned in a small section of the electrophoresis capillary or forms part of an external device that is coupled to the electrophoresis capillary [4,14–21]. Evidently, these systems have higher sample loadability compared to sophisticated sample injection techniques such as field-amplified stacking and transient isotachophoresis, since the loading capacity is not limited by the total capillary volume [19].

Preconcentration methods can be classified as non-selective and selective, depending on the affinity of the solid support for the analytes [8]. For selective analyte preconcentration, on-line immunoaffinity capillary electrophoresis has found widespread application. In immunoaffinity capillary electrophoresis, specific antibodies bound to the surface of a porous material (e.g., porous polymer, glass beads, silica beads, membrane, or the capillary wall itself) are used for the selective concentration of specific antigens [5,8,14,22]. Following capture, the antigens are eluted with a small plug of an elution buffer that disrupts the binding affinity. The desorbed antigens are then separated by CE.

Several groups have achieved on-line preconcentration CE using up to 1 cm solid packing material placed near the inlet of the separation capillary, and kept in position with frits. In addition to the formation of bubbles, a major disadvantage of this design is the increased back-pressure generated by the use of frits, which disrupts the electroosmotic flow (EOF) and eventually induces blockage of the capillary. To alleviate this problem, the use of a magnet instead of frits to hold the solid packing in place has been proposed [16]. Another approach is to replace the solid phase preconcentrator by an open tubular preconcentrator. Guzman [20] reported the use of antibodies immobilized on the wall of an array of open tubular capillaries attached to the separation capillary for the selective preconcentration of IgE. An advantage of this design is the absence of frits and packing materials.

More recently, in a very elegant approach, Guzman and Phillips [15,23] introduced an improved solid-phase microextraction system for use in on-line immunoaffinity CE. The system had a cross-shaped configuration, connecting the solid-phase extractor to two large-bore capillaries for sample and buffer transport, and to two small-bore capillaries for CE.

Polymeric materials have also been proposed as absorptive phases. Several groups have reported the use of membranes for preconcentration in CE [5,24–26]. This technique is termed “on-line membrane preconcentration-CE” and is based on the use of a polymeric membrane that is sandwiched in between two capillaries. An advantage of this approach is that because the preconcentration capillary can be separated from the separation capillary during sample loading, there is more flexibility in buffer selection. In addition, buffer and sample introduction becomes easy. A limitation, however, is the need to couple the preconcentration and separation capillaries. It is a fact that innovative designs must be found to alleviate the limitations of previously reported preconcentrators.

A versatile chromatographic support termed a monolith was first introduced in 1989 by Hjertén et al. [27]. Polymer monoliths [28,29] are typically prepared by in situ polymerization of monomer solutions composed of a monomer, crosslinker, porogen and initiator. Polymerization is initiated either thermally or by UV light. Because of the flexibility in monomer choice, monoliths with a variety of surface chemistries can be prepared [30]. An attractive feature that makes monoliths amenable as chromatographic supports is that no frits are required, since the rods are directly synthesized within the column [30]. The highly porous structures of monolithic columns give them high mechanical strength, low flow resistance and high rates of mass transfer. Diffusion in monoliths is much faster than in conventional supports and is no longer a limiting factor for analyte interaction. Consequently, the use of high flow rates is possible and rapid separations result [30].

The potential of monoliths as stationary phases for biochromatography has been extensively demonstrated. Acrylate, methacrylate and styrene based monoliths have been around since the early 1990s. The applicability of GMA monoliths in affinity chromatography for analytical and preparative purification of proteins has been demonstrated [30]. Polymeric monoliths molded within microfluidic devices have been used for on-chip solid-phase extraction of a standard peptide and green fluorescent protein [31]. More recently, the use of methacrylate based monoliths in capillary electrophoresis for the selective preconcentration of *S*-propanolol was demonstrated by Baryla and Tolt [18].

Here we propose the use of a polymeric monolith as the support for protein preconcentration prior to CE. To the best of our knowledge, only one system similar to the one proposed here has been reported [18]. However, it was not applied to the preconcentration of proteins, analysis of real samples was not demonstrated, and surface passivation of the fused silica capillary was not done. Furthermore, the porous properties of the monolith were not reported. Design, characterization and evaluation of an on-line protein G monolithic preconcentration-CE system for enrichment and separation of proteins is described in this paper.

2. Experimental

2.1. Chemicals

Anhydrous methanol, acetone and hexanes were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Cyclohexanol was from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was from Anachemia Canada (Montréal, Canada). Dextran sulfate sodium salt, hexadimethrin bromide (polybrene), glycidyl methacrylate (GMA) 97%, 3-(trimethoxysilyl)propyl methacrylate (γ-MPTS), trimethylolpropane trimethacrylate (TRIM) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) 99% were supplied

by Sigma–Aldrich (Milwaukee, WI, USA). Ammonium formate and phosphate buffered saline (PBS) 10× solution (pH 7.4 ± 0.1) were also supplied by Fisher Scientific. Sodium carbonate monohydrate and sodium bicarbonate were from EM Science (Darmstadt, Germany). Protein G, recombinant *Escherichia coli*, and human immunoglobulin G (IgG) were purchased from Calbiochem (La Jolla, CA, USA). Buffer solutions were prepared with deionized water from a Millipore water purifier (Molsheim, France) and filtered through a 0.22 μm filter.

2.2. Capillary zone electrophoresis

Fused silica capillary tubing with 75 μm I.D. and 365 μm O.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). Capillary electrophoresis (CE) experiments were performed with a Crystal CE 300 system (ATI, Madison, WI, USA) equipped with an online Crystal 100 variable wavelength UV–vis absorbance detector and a Chrom Perfect software work station (Mountain View, CA) for data collection and treatment. On-column UV detection was performed at 214 nm. Electropherograms were converted to a CP Tab delimited raw file with RT and redrawn using Microsoft Excel (Redmond, WA, USA).

2.3. Monolithic preconcentrator design and evaluation

2.3.1. Capillary surface deactivation

Capillary surface deactivation was accomplished by depositing alternating thin films of physically adsorbed negatively (dextran) and positively (polybrene) charged polymers. We applied a methodology similar to that described by Katayama et al. [32] with some modifications. Briefly, using a syringe pump (Model 11, Harvard Apparatus, Holliston, MA, USA) with a 1 mL plastic syringe fitted with a stainless steel needle, a 75 μm fused silica capillary was washed consecutively with acetone, deionized water, 0.2 M HCl, deionized water, 0.2 M NaOH and deionized water for 30 min each at 10 μL/min. The capillary was then rinsed with a 10% polybrene solution at 5 μL/min for 30 min and allowed to sit for 15 min. Next, the capillary was washed with a 6% dextran solution at 5 μL/min for 30 min and left for 15 min. Finally, the capillary was rinsed again with 10% polybrene solution at 5 μL/min for 30 min and left for 1 h. Non-adsorbed polymer was washed away with H_2O.

2.3.2. Preparation of polymer monoliths

The surface deactivated capillary was dried using a stream of nitrogen for 1 h. At 5 cm from the inlet end, a 2 cm length of the capillary was etched with concentrated sulfuric acid to remove the outer polyimide coating to generate a UV transparent window for polymerization of the monolith. The monomer mixture was prepared in a 1 dram (4 mL) glass vial by admixing in sequence DMPA (initiator), TRIM (cross-linker), GMA (monomer) and cyclohexanol, methanol and hexane (porogens), and ultrasonicating for 5 min before use. This monomer solution was introduced into the capillary by capillary action. Polymerization in the UV transparent region of the capillary was induced using a Dymax 5000 AS UV curing lamp (Torrington, CT, USA). The UV curing lamp can produce an irradiation intensity of 200 mW/cm^2 in the wavelength range of 320–390 nm. The irradiation time was varied from 8 to 15 min. Unreacted monomer and porogens were flushed out of the capillary by rinsing with 1 mL of methanol. More details on the composition of the reagent solution for various monoliths used in this study for protein preconcentration are provided in Section 3.1.2.

2.3.3. Immobilization of protein G on polymer monoliths

Protein G was immobilized on GMA monolithic columns following a procedure similar to that described by Tennikova and co-workers [33]. Briefly, using a syringe pump (Model 11, Harvard Apparatus, Holliston, MA, USA) with a 1 mL plastic syringe fitted with a stainless steel needle, the monolithic capillary column was washed consecutively with ethanol, ethanol–H_2O (1:1), H_2O and 0.1 M sodium carbonate buffer (pH 9.3) for 30 min each at 2 μL/min. A solution of 5.0 mg/mL protein G dissolved in 0.1 M sodium carbonate buffer (pH 9.3) was then pumped through the monolithic column for 20 min at 0.4 μL/min. Using silicone rubber, both ends of the capillary were sealed. The monolithic column was then heated to 34 °C for 20 h in an oven. Noncovalently bound protein was washed away with 0.1 M sodium carbonate buffer (pH 9.3), followed by 50 mM ammonium formate–formic acid buffer, pH 7.6. The monolithic column was stored in this last buffer at 4 °C until used.

2.3.4. Detection window preparation

The protein G monolithic preconcentrator capillaries were cut in lengths of 64 cm, and a detection window was burned at 53 cm from the inlet end.

2.4. Scanning electron microscopy (SEM)

An aliquot of 0.3 g of optimized monolithic precursor solution, prepared as outlined in Section 2.3.2, was dispensed into a 1 dram (4 mL) glass vial and irradiated under the UV lamp for 8 min. The bulk monolith was carefully removed by breaking the glass vial, cut into peaces with a razor blade, Soxhlet extracted with methanol overnight and placed in a vacuum oven at 60 °C overnight. The dry monolith was used to obtain SEM images. The monolith was sputtered with ∼20 nm gold, and SEM images were taken using an FEI Philips XL30 ESEM FEG (Hillsboro, OR, USA).

2.5. Porous properties

The physical properties (specific surface area, average pore diameter and porosity) of the bulk monolith were determined by mercury intrusion porosimetry using an Auto Pore IV 9500 V1.03 (Micromeritics, Norcross, GA, USA).

2.6. Capillary liquid chromatography

To investigate the influence of the monolith formulation on binding capacity, and to evaluate the effect of the speed of sample application on protein adsorption, affinity LC experiments

were conducted. Capillary LC experiments were performed using a syringe pump (Model 11, Harvard Apparatus, Holliston, MA, USA) with a 25 μL Hamilton gastight syringe (Reno, NV, USA) fitted with a stainless steel needle, a Linear Scientific UV–vis 203 detector (Reno, NV, USA), and a Thermo Separations PC1000 V3.0 software work station (Fremont, CA, USA) for data collection and treatment. Affinity LC experiments were performed as follows. The capillary was conditioned with PBS for 5 min at 1 μL/min. Following injection of IgG solution (in which IgG was dissolved in PBS) for a set amount of time at a set flow rate, the capillary was sequentially rinsed with PBS for 5 min at 1 μL/min and 20 mM HCl at 0.5 μL/min. Eluted IgG was detected at 214 nm. The total length of the capillary was 20 cm and the effective length was 15 cm.

2.7. On-line preconcentration-CZE of IgG

On-line preconcentration-CZE of IgG was achieved as follows. First, the protein G monolithic preconcentrator capillary was conditioned with 50 mM ammonium formate–formic acid buffer, pH 7.6 (binding buffer) for 6 min at 1 bar. An IgG solution was then loaded for a set amount of time at 1 bar. Unbound protein was washed away by rinsing with binding buffer for 6 min at 1 bar. Following preconditioning of the capillary with 12.5 mM ammonium formate–formic acid, pH 7.6 (separation buffer), IgG was desorbed from the protein G monolith by injecting a small plug (equivalent to three times the monolith length, ∼1 bar for 0.3 min) of 50 mM formic acid (elution buffer). A plug of equal length of separation buffer was next injected. Eluted IgG was electrophoresed along the separation capillary and detected by UV absorption. The applied voltage was 15 kV and the detection wavelength was 214 nm.

2.8. On-line extraction and preconcentration of IgG from human serum

Venous blood was obtained from a healthy volunteer. Blood samples were collected in a Greiner Bio-one Vacuette tube containing Z Serum Sep. clot activator (Longwood, FL, USA) and centrifuged at 4 °C at 3600 rpm for 12 min. Separated serum was stored at −80 °C until used. Thawed serum samples were diluted 1:10 in 50 mM ammonium formate–formic acid (pH 7.6), sonicated for 20 s and heated at 95 °C for 5 min [34].

3. Results and discussion

3.1. Monolithic preconcentrator design and evaluation

3.1.1. Capillary surface deactivation

It became evident early in the experiments that non-specific adsorption of proteins to the surface of the capillary could be problematic. Initial experiments were performed using a capillary with an inner surface that had previously been treated with γ-MPTS to ensure covalent bonding of the monolith to the capillary wall. Unfortunately, this capillary soon proved to non-specifically adsorb proteins during the preconcentration step (see Section 3.2). This phenomenon was not surprising since nonspecific interactions (van der Waals, hydrophobic and electrostatic) between proteins and surfaces always exist. Permanent coating of the surface of the capillary with polymers that are either covalently bonded or physically adsorbed to the surface of the capillary may be the most effective way of deactivation [32]. Accordingly, the first step in the design of the analyte preconcentrator-CE system involved the deactivation of the fused silica capillary surface. Protein compatibility and coating stability were two of the criteria for selection of the polymeric materials. Additionally, stability of the monolith within the coated capillary had to be ensured.

Katayama et al. [32] developed a method to coat fused silica capillaries with successive multiple ionic polymer layers of polybrene and dextran. Highly efficient separations with good reproducibility were reported using this methodology. Additionally, the coating proved chemically stable and useful for real biological sample analysis. We applied a similar approach to passivate the surface of the fused silica capillary prior to casting of the monolith. It should be mentioned that even though the polymer monolith was not explicitly covalently bound to the capillary wall as in many other studies describing monoliths for CEC, the monolith is very stable and did not move at all within the capillary when pressure or high voltage was applied. We believe that an electrostatic mechanism holds the monolith to the positively charged PB(3) coated capillary. As discussed later in Section 3.2, measurements of the EOF of the GMA monolithic capillaries revealed the presence of negative charge on the monolithic support.

Human IgG, with very high affinity towards protein G, was the protein of choice to evaluate the protein G monolithic preconcentrator. Following capillary surface deactivation, appropriate CE separation conditions for IgG were determined. The challenge here was to find a discontinuous background electrolyte system compatible with both steps, preconcentration and CE. Several experiments were run using a combination of discontinuous buffer systems. Acetate, borate, tris and formate were among the background electrolyte buffers tested. Acetic acid, HCl–glycine and formic acid were among the sample buffers tested. A suitable discontinuous buffer for the CE of IgG was determined to be 12.5 mM ammonium formate–formic acid (pH 7.6) as the background electrolyte and 50 mM formic acid as the sample buffer.

3.1.2. Monolith preparation

A series of experiments were conducted to produce monoliths with the required characteristics (high surface area, homogeneity and low back-pressure). A reliable method to prepare monolithic preconcentrators was developed. GMA was selected as the monomer since it provides monoliths with epoxy groups to which amine groups present in proteins can be immobilized.

An ideal monolith for protein preconcentration should have high surface area and low flow resistance. While the surface area mainly comes from the contribution of micropores and mesopores in the skeletal structure, the pressure drop is determined by the medium pore diameter of the throughpores. Unfortunately, in most cases, the medium pore diameter is correlated with the surface area in a polymer monolith. For example, high surface areas

are often accompanied by small throughpores, which results in a concomitant increase in flow resistance. Thus, a balance between surface area and flow resistance must be made. Among the variables to adjust the pore size distribution of a polymer monolith, porogen and initiation technique are the most effective.

For the preparation of a suitable poly(GMA-co-TRIM) monolith, a variety of porogens were considered. These ranged from long-chain alcohols, such as cyclohexanol and dodecanol [30], to low boiling point organic solvents, such as toluene and isooctane [35]. Since the pore size distribution of a polymer monolith is also strongly dependent on the initiation technique used (e.g., thermal versus UV versus redox), it is not surprising that an optimized recipe developed by one research group cannot be directly implemented by another group without modification. This was found true in our experiments, and we observed that the optimized recipes developed by Hjertén et al. [27] and Viklund et al. [35] could not yield a monolith with sufficiently low pressure drop to be used in our CE instrument (1 bar for ∼2 cm monolith). Thus, new porogens were sought in order to develop a uniform poly(GMA-co-TRIM) monolith with extremely low flow resistance.

To design the porogen system, two variables were kept constant. In all experiments, the initiator (DMPA) concentration relative to total monomer (GMA + TRIM) concentration was kept at 1%. In addition, the GMA to TRIM ratio was fixed at 60:40 (wt%). Six organic solvents (cyclohexanol, dodecanol, toluene, isooctane, methanol and hexane) were investigated, and classified into three categories based on the final pressure drop of the monolith prepared with the pure organic solvent as porogen. Toluene was classified as a microporogen; cyclohexanol and dodecanol as mesoporogens; and methanol, hexane and isooctane as macroporogens. The final optimized porogen, ensuring macroscopically homogeneous monoliths with low flow resistance (∼0.6 μL/min at 1 bar for a 2 cm monolith), was determined to be a ternary system composed of methanol, hexane and cyclohexanol. Several optimized recipes based on this ternary porogen are listed in Table 1.

Protein G was immobilized on monoliths prepared according to the recipes listed in Table 1. These protein G monoliths were used to investigate the effect of monolith formulation on binding capacity. To determine the binding capacity of the monolithic preconcentrator, the protein G monolith was saturated with an IgG solution and the area of the eluted peak was measured. All experiments were performed using affinity capillary liquid chromatography (see Section 2.6).

Fig. 1 shows the influence of monolith formulation on binding capacity. From Fig. 1, for the monolith formulations studied, there was no significant effect of the composition of the monolithic precursor solution on binding capacity. Therefore, monolith rod 2, with the lowest back-pressure, was selected for further characterization.

Table 1
Compositions of the optimized monolithic preconcentrator formulations used in this study to evaluate the effect of monolith formulation on binding capacity[a]

Monolith	DMPA	TRIM	GMA	Cyclohexanol	Methanol	Hexane
1	0.005	0.20	0.30	1.12	0.26	0.11
2	0.006	0.24	0.36	0.77	0.44	0.19
3	0.008	0.32	0.32	0.18	0.71	0.30

[a] Units are in grams.

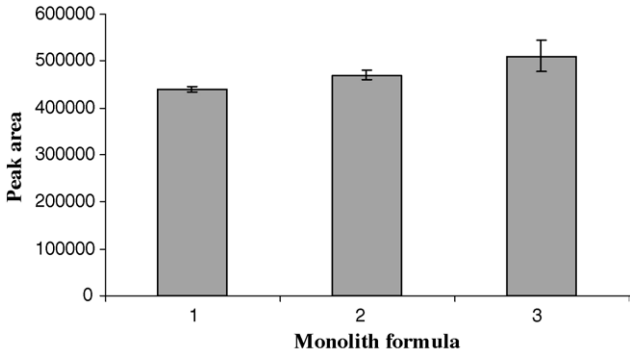

Fig. 1. Influence of monolith formulation on binding capacity. Experimental conditions: 20 cm (15 cm to detector) × 75 μm I.D. fused silica capillary; 2 cm protein G monolithic preconcentrator; 2 mM HCl (0.5 μL/min) elution buffer; UV detection at 214 nm; 300 μg/mL IgG sample; 1 μL/min sample loading flow rate; 15 min sample loading time. The recipes for all of the monoliths are listed in Table 1.

3.1.3. Determination of the physical properties of GMA monolith rod 2

The physical properties of GMA monolith rod 2 were determined using a mercury intrusion porosimeter. Fig. 2 shows the pore size distribution of this monolith. The total pore area was 4.79 m^2/g, the average pore diameter was 3.29 μm and the porosity was 82%. These account for the low flow resistance of this monolith. An SEM image of this monolith is provided in Fig. 3.

3.1.4. Evaluation of the effect of speed of sample application on protein adsorption

One of the attributes of monolithic supports is their high rates of mass transfer and low flow resistance, which makes fast analysis possible. Therefore, the influence of speed of sample application on protein adsorption was investigated. During the sample loading step, flow rates were varied from 0.5 to 10 μL/min to demonstrate the ability of the monolith to rapidly preconcentrate proteins. All experiments were performed using affinity capillary liquid chromatography (see Section 2.6); in all cases, saturation of the protein G monolith occurred.

The influence of speed of sample application on peak area of retained IgG was determined by loading a constant volume (15 μL) of a 300 μg/mL IgG solution at varying flow rates and

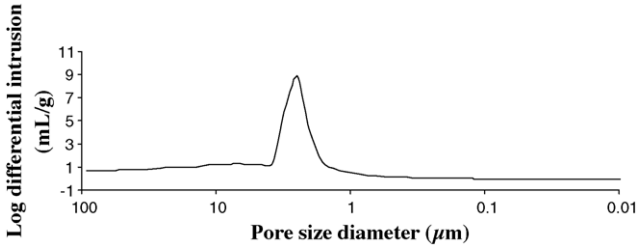

Fig. 2. Pore size distribution of GMA monolithic rod 2 measured by mercury intrusion porosimetry.

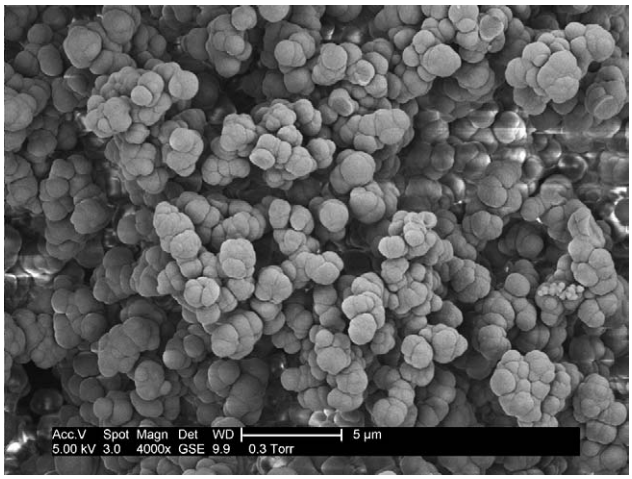

Fig. 3. SEM photograph of a monolithic capillary column.

measuring the area of the eluted peak. When the flow rate was varied from 0.5 to 10 μL/min, no significant change was observed in the area of the eluted peak (data not shown), indicating the ability of the monolith to rapidly preconcentrate proteins.

3.2. Method development for on-line preconcentration-CE of IgG

In Section 3.1.1, it was discussed that capillaries treated with γ-MPTS strongly adsorbed IgG during the preconcentration step. This was not the case for PB(3) coated capillaries.

Fig. 4 demonstrates the effectiveness of PB(3) to prevent non-specific adsorption of IgG on the surface of the capillary during the preconcentration step. To obtain Fig. 4, a monolithic preconcentrator capillary lacking protein G was used; the baseline was monitored during the preconditioning, loading, washing and elution steps. As seen from Fig. 4A, the capillary treated with γ-MPTS exhibited a peak at approximately 60 min. The capillary coated with PB(3) (Fig. 4B), on the other hand, did not show such a peak. These results suggest that during the preconcentration step, IgG is non-specifically adsorbed on the surface of the capillary treated with γ-MPTS, after which it is released as the capillary is rinsed with the elution buffer. This is evidence that PB(3) effectively suppresses the non-specific adsorption of IgG on the capillary wall. To further demonstrate the effectiveness of PB(3) to eliminate protein adsorption, the EOF of the PB(3) coated capillary was measured before and after flushing the capillary with an IgG solution. No significant change in the EOF was observed (data not shown).

Interestingly, the EOF of the PB(3) coated capillary was reversed from anodic to cathodic upon casting of the GMA monolith as observed in Fig. 5. Fig. 5A is a typical electropherogram of a neutral marker (DMSO) run on a PB(3) coated capillary, while Fig. 5B is an electropherogram of the same marker run on a GMA monolithic capillary lacking protein G. A cathodic EOF was still observed for the protein G monolithic capillary (data not shown), which reveals the presence of negative charges on the monolithic support. Adsorption of buffer ions from the buffer solution have been observed to produce a

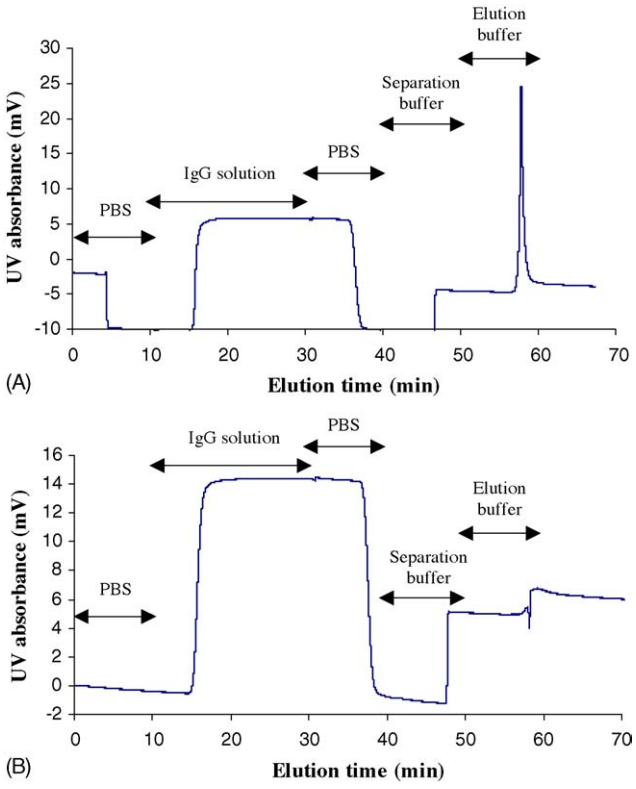

Fig. 4. Baseline monitoring of the affinity LC experiment with IgG as sample solution to test the non-specific adsorption of protein on the surface deactivated fused silica capillary. Experimental conditions: 75 cm (64 cm to detector) × 75 μm I.D. fused silica capillary; 2 cm protein G monolithic preconcentrator. The column was flushed with the binding buffer (PBS) for 10 min at 1 bar, after which a 100 μg/mL IgG solution was injected for 20 min at 1 bar. After injection, the column was flushed consecutively with binding buffer (PBS), separation buffer and an elution buffer for 10 min each at 1 bar. Monolithic preconcentrator without protein G: (A) γ-MPTS treated capillary and (B) polybrene coated capillary.

negative zeta potential on Teflon, PP and PMMA column surfaces [36,37]. Therefore, The cathodic EOF was hypothesized to stem from either the presence of impurities (methacrylic acid) in the monomers used to prepare monolithic precursor solutions, or adsorption of buffer ions on the monolithic support. Accordingly, CE of IgG on protein G monolithic preconcentrators was performed using a positive voltage polarity.

It should be mentioned that the separation of IgG after preconcentration on protein G monolithic preconcentrators was only accomplished when the plug of elution buffer (containing the desorbed IgG) was followed by an injection of separation buffer (12.5 mM ammonium formate–formic acid, pH 7.6) sufficient to cover the monolith. Failure to inject separation buffer resulted in re-adsorption of the desorbed IgG on the protein G monolith regardless of voltage polarity. Considering that the charge of IgG is dependent on the pH of the medium and that IgG bears a positive charge when dissolved in elution buffer, re-adsorption was believed to stem from electrostatic interactions between the negatively charged protein G monolith and the positively charged IgG.

Fig. 6 illustrates the steps of on-line preconcentration-CE of IgG. IgG standards at different concentrations (120 and 12 nM)

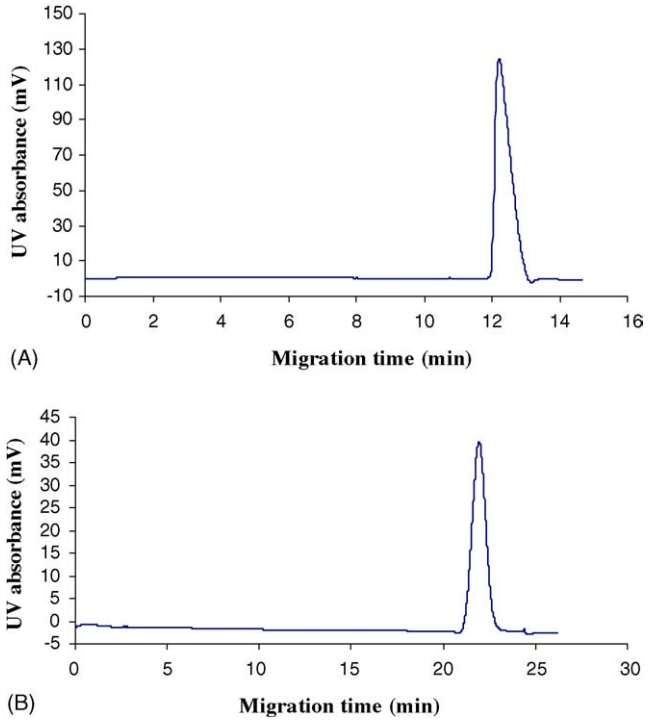

Fig. 5. Electropherogram of IgG demonstrating the reversal of the EOF upon casting a GMA monolith inside a polybrene coated fused silica capillary. Experimental conditions: 70 cm (57 cm to detector) × 75 μm I.D. fused silica capillary; neutral marker (DMSO) as sample; 50 mM ammonium formate–formic acid (pH 7.6) separation buffer; UV detection at 214 nm. (A) Polybrene coated capillary, −15 kV applied separation voltage and (B) 1.5 cm GMA monolith cast inside a polybrene coated capillary, +15 kV applied separation voltage.

were preconcentrated using this system (electropherograms not shown). It is noteworthy to mention that preconcentration of IgG at concentrations lower than 12 nM is possible, and that the lowest sample concentration that can be detected with this system (or the CLOD of this system) is ultimately determined by the volume of sample injected. Preconcentration of IgG at lower concentrations was demonstrated using more complex samples, such as human serum, which will be discussed later in Section 3.3.

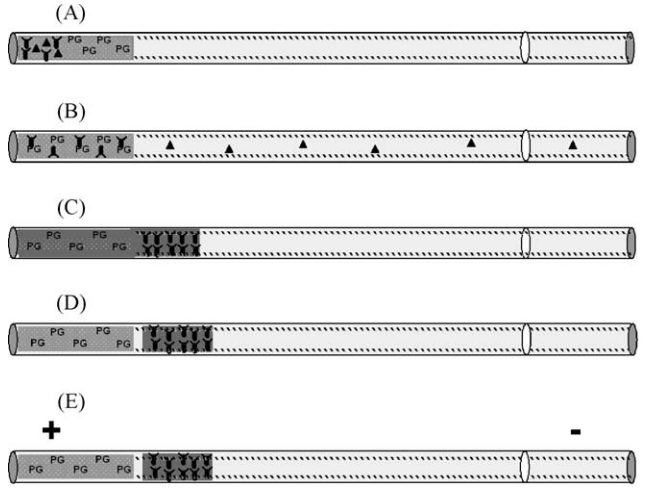

Fig. 6. Schematic representation of the steps of on-line preconcentration-CE of IgG: (A) sample injection following preconditioning of the protein G-monolithic preconcentrator capillary with 50 mM ammonium formate–formic acid (pH 7.6), (B) removal of unbound proteins and preconditioning of the preconcentrator with 12.5 mM ammonium formate–formic acid (pH 7.6), (C) desorption of trapped IgG with 50 mM formic acid, (D) injection of a plug of 12.5 mM ammonium formate–formic acid (pH 7.6) and (E) electrophoresis.

On-line preconcentration-CE of IgG was fairly reproducible. For the preconcentration of a 1.2 μM IgG solution, the average migration time and peak area were 8.46 ± 0.45 min and $(1.2 \pm 0.062) \times 10^6$ μV s, respectively. These results were calculated based on three measurements. Slight variations in migration times can be attributed to protein precipitation issues during the freezing and thawing cycles. Additionally, in the course of the preparation of the monolithic preconcentrator, slight changes in the porogen content in the monolithic precursor solution may have led to monoliths with slightly different average pore size and, therefore, different back-pressure and surface area of charged groups, which ultimately affected the net EOF.

3.3. Application of the monolithic preconcentrator to a human serum sample

The ability of the on-line monolithic preconcentrator-CZE to preconcentrate IgG was proven for real samples. Diluted (500 and 65,000 times) human serum samples obtained from a healthy volunteer were analyzed using this system. Assuming a 10–15 mg/mL IgG concentration in human serum, for a

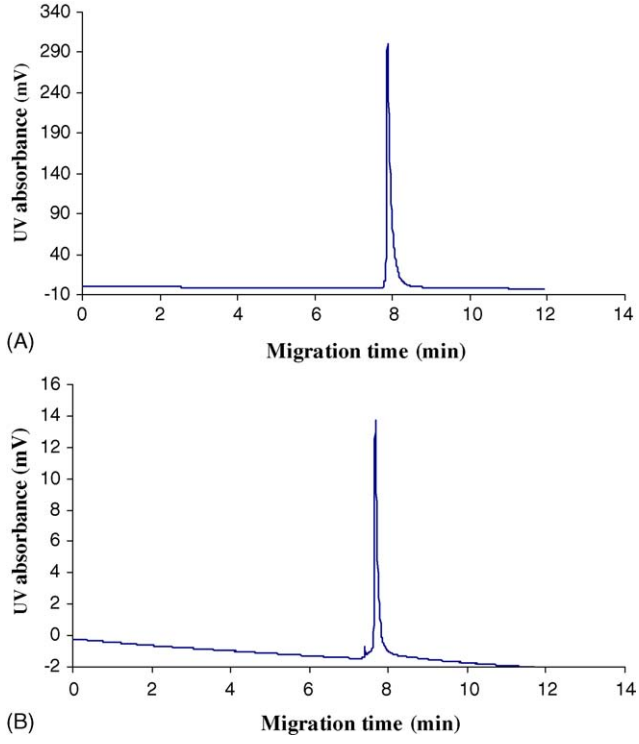

Fig. 7. Electropherograms demonstrating on-line preconcentration-CE of IgG from human serum. Experimental conditions: 64 cm (53 cm to detector) × 75 μm I.D. fused silica capillary; 1.5 cm protein G monolithic preconcentrator; 50 mM formic acid (1.0 bar, 0.3 min) elution buffer; 12.5 mM ammonium formate–formic acid (pH 7.6) separation buffer; +15 kV applied separation voltage; UV detection at 214 nm. (A) 500 times diluted human serum (∼6 μL volume sampled) and (B) 65,000 times diluted human serum (∼28 μL volume sampled).

65,000 diluted serum sample, the IgG concentration was estimated to be 960 pM to1.4 nM. Typical electropherograms of IgG extracted and preconcentrated from human serum are shown in Fig. 7. These results demonstrate the effectiveness of the preconcentrator for on-line preconcentration-CE of proteins in real biological samples. In this particular application, the preconcentrator served three purposes: sample extraction, clean-up and preconcentration. The system could be used for more than eight consecutive runs without significant loss in performance (average migration time and peak area were 8.29 ± 0.48 min and $(4.03 \pm 0.30) \times 10^5$ μV s, respectively). The system was stable for more than one month upon storage.

It is noteworthy to mention that this preconcentrator is not limited to the preconcentration of IgG. By having protein G immobilized on the monolith, a universal support is generated that allows the capture of any protein for which an antibody is available. This study was particularly focused on the design and evaluation of a preconcentrator that could be used for the selective preconcentration of a wide range of proteins. IgG was the standard protein chosen to test this preconcentrator. The application of this device for the preconcentration of serum proteins that can be used as biomarkers, and its usefulness for quantitative purposes is the subject of a future publication. This preconcentrator can potentially be coupled to MS, further lowering the CLOD.

4. Conclusions

An on-line protein G monolithic preconcentrator-CE system was designed and evaluated for the preconcentration of proteins. In fabricating the preconcentrator, the use of coated capillaries was essential to reduce protein-wall interactions. In addition to providing good separation efficiencies, PB(3) coated capillaries proved effective to prevent the non-specific adsorption of IgG on the surface of the capillary during the preconcentration step. Monoliths from different formulations were prepared and evaluated for binding capacity to optimize the monolith formulation for protein preconcentration. The physical properties of the monolith considered best were determined by mercury intrusion porosimetry. The monolith had a low back-pressure and was macroscopically homogeneous. The potential of the monolith to rapidly preconcentrate proteins was demonstrated. A suitable discontinuous buffer system for on-line preconcentration-CE separation of IgG was determined to be 12.5 mM ammonium formate–formic acid (pH 7.6) as the background electrolyte and 50 mM formic acid as the elution buffer. Standard solutions of IgG were preconcentrated using this system. Additionally, the extraction and preconcentration of IgG from human serum was demonstrated. The on-line preconcentration-CZE system developed here can potentially be applied to the analysis of proteins other than IgG for which an antibody is available; therefore, it should be very useful for the analysis of biomarkers, which are usually proteins that are present at low concentrations.

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References

[1] T. Manabe, Electrophoresis 21 (2000) 1116.
[2] J.A. Wells, H. Swerdlow, Anal. Chem. 75 (2003) 5207.
[3] W.L. Tseng, H.T. Chang, Anal. Chem. 72 (2000) 4805.
[4] M.A. Strausbauch, J.P. Landers, P. Wettstein, Anal. Chem. 68 (1996) 306.
[5] A.J. Tomlinson, L.M. Benson, N.A. Guzman, S. Naylor, J. Chromatogr. A 744 (1996) 3.
[6] A.J. Tomilinson, S. Naylor, J. Capillary Electrophor. 5 (1995) 225.
[7] A.J. Tomlinson, N.A. Guzman, S. Naylor, J. Capillary Electrophor. 6 (1995) 247.
[8] J.J. Dalluge, L.C. Sander, Anal. Chem. 70 (1998) 5339.
[9] N.A. Guzman, R.J. Stubbs, Electrophoresis 22 (2001) 3602.
[10] N.A. Guzman, M.A. Trebilcock, J.P. Advis, J. Liq. Chromatogr. 14 (1991) 997.
[11] N.H. Heegaard, S. Nilsson, N. Guzman, J. Chromatogr. B 715 (1998) 29.
[12] T. Stroink, E. Paarlber, J.C.M. Waterval, A. Bult, W.J.M. Underberg, Electrophoresis 22 (2001) 2374.
[13] Q. Yang, A.J. Tomlinson, S. Naylor, Anal. Chem. 71 (1999) A183.
[14] N.A. Guzman, J. Chromatogr. B 749 (2000) 197.
[15] N.A. Guzman, Electrophoresis 24 (2003) 3718.
[16] L. Rashkovetsky, Y. Lyubarskaya, F. Foret, D. Hughes, B. Karger, J. Chromatogr. A 781 (1997) 197.
[17] A.M. Hoyt, S.C. Beale, J.P. Larmann, J.W. Jorgenson, J. Microcol. Sep. 5 (1993) 325.
[18] N.E. Baryla, N.P. Tolt, Analyst 128 (2003) 1009.
[19] J.C.M. Waterval, G. Hommels, J. Teeuwsen, A. Bult, H. Lingeman, W.J.M. Underbeg, Electrophoresis 21 (2000) 2851.
[20] N.A. Guzman, J. Liq. Chromatogr. 18 (1995) 3751.
[21] J. Cai, Z. El Rassi, J. Liq. Chromatogr. 16 (1993) 2007.
[22] J.P. Quirino, M.T. Dulay, B.D. Bennet, R. Zare, Anal. Chem. 73 (2001) 5539.
[23] N.A. Guzman, T.M. Phillips, Anal. Chem. (2005) 61A.
[24] E. Rohde, A.J. Tomlinson, D.H. Johnson, S. Naylor, J. Chromatogr. B 713 (1998) 301.
[25] R. Lehmann, W. Voelter, H.M. Liebich, J. Chromatogr. B 697 (1997) 3.
[26] N.A. Guzman, S.S. Park, D. Schaufelberger, L. Hernandez, X. Paez, P. Rada, A.J. Tomlinson, S. Naylor, J. Chromatogr. B 697 (1997) 37.
[27] S. Hjertén, J.L. Liao, R. Zhang, J. Chromatogr. 473 (1989) 273.
[28] A. Palm, M.V. Novotny, Anal. Chem. 69 (1997) 4499.
[29] F. Svec, J.M.J. Fréchet, Anal. Chem. 54 (1992) 820.
[30] Z. Pan, H. Zou, W. Mo, X. Huang, R. Wu, Anal. Chim. Acta 466 (2002) 141.
[31] C. Yu, M.H. Davey, F. Svec, J.M.J. Frechet, Anal. Chem. 73 (2001) 5088.
[32] H. Katayama, Y. Ishihama, N. Asakawa, Anal. Chem. 70 (1998) 5272.
[33] T.V. Gupalova, O.V. Lojkina, V.G. Pàlàgnuk, A.A. Totolian, T.B. Tennikova, J. Chromatogr. A 949 (2002) 185.
[34] Y.Y. Wang, P.C.P. Cheng, D.W. Chan, Proteomics 3 (2003) 243.
[35] C. Viklund, E. Ponten, B. Glad, K. Irgum, Chem. Mater. 9 (1997) 463.
[36] K.D. Lukacs, J.W. Jorgenson, J. High Resolut. Chromatogr. Chromatogr. Commun. 8 (1985) 407.
[37] S. Chen, M. Lee, J. Microcol. Sep. 9 (1996) 57.