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ScienceDirect
Journal of Chromatography B, 841 (2006) 152–159
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Capillary isoelectric focusing of proteins and microorganisms in dynamically modified fused silica with UV detection☆

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Received 7 February 2006; accepted 8 May 2006
Available online 9 June 2006

Abstract

We suggest a method for the reproducible and efficient capillary isoelectric focusing of proteins and microorganisms in the pH gradient 3–10. The method involves the segmental injection of the simple ampholytes, the solution of the selected electrolytes, and the sample mixture of bioanalytes and carrier ampholytes to the fused silica capillaries dynamically modified by poly(ethylene glycol), PEG 4000, which is added to the catholyte, the anolyte and injected solutions. In order to receive the reproducible results, the capillaries were rinsed by the mixture of acetone/ethanol between analyses. For the tracing of the pH gradients the low-molecular-mass p*I* markers were used. The simple proteins and the mixed cultures of microorganisms, *Saccharomyces cerevisiae* CCM 8191, *Escherichia coli* CCM 3954, *Candida albicans* CCM 8180, *Candida parapsilosis*, *Candida krusei*, *Staphylococcus aureus*, *Streptococcus agalactiae* CCM 6187, *Enterococcus faecalis* CCM 4224, *Staphylococcus epidermidis* CCM 4418 and *Stenotrophomonas maltophilia*, were focused and separated by the method suggested. The minimum detectable number of microbial cells was 5×10^2 to 1×10^3 with on-column UV detection at 280 nm.

Keywords: CIEF; Dynamically modified FS; Spacers; p*I* markers; Proteins and microorganisms; UV detection

1. Introduction

The application of the electromigration techniques [1] in the diagnostic microbiology needs the reliable method, the high run-to-run reproducibility, speed and sensitivity. The wide spectrum of physiological features [2] makes the characterization of microbes by capillary zone electrophoresis (CZE) difficult. The microbes are different in their size, shape and in the composition of the outer membranes, which include the large number of lipids, proteins, etc. The isoelectric points, p*I*'s, of microbes [3] are more appropriate parameters than the electrophoretic mobilities; p*I*'s are practically independent from the measurement conditions [4,5]. Several papers were published showing the successful separation of microorganisms according to their p*I* by capillary isoelectric focusing (CIEF) [1,5,6].

Microorganisms, similarly to bioanalytes, tend to adhere strongly on the surfaces [7,8]. The adsorption of the analytes onto the capillary wall may increase the zone distortion [1,9–11]. Simultaneously, it is necessary to minimize the strong electroosmotic flow (EOF) on the uncoated fused silica (FS) capillary. The suggested solutions include dynamic modification of the inner capillary surface [12,13] by soluble polymers such as hydroxymethylpropyl cellulose, hydroxypropyl cellulose, polyvinyl alcohol, polyacrylamide, poly(acryl-amide-*co*-acrylic acid), polyvinylpyrrolidone, etc. [12,14,15]; coating of the capillary with the hydrophilic polymer such as an acrylamide derivative; and modification of the capillaries by γ-glycidoxypropyltrimethoxysilane [16,17] or the sol–gel technique [18]. The lifetime of the polymeric film on the inner surface of the capillary is often shortened by its degradation due to the strong acids and bases used as the anolytes and catholytes, respectively [12]. At the same time, the adsorbed bioanalytes must be washed out from the wall of the capillary prior to the subsequent run. The rinsing procedure between individual runs was shown to have a strong effect on the reproducibility [19]. Therefore, the application of the

☆ This paper is part of a special volume entitled "Analysis of proteins, peptides and glycanes by capillary (electromigration) techniques", dedicated to Zdenek Deyl, guest edited by I. Miksik.

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doi:10.1016/j.jchromb.2006.05.013

uncoated dynamically modified FS seems to be the simplest solution.

The poly(ethylene oxide) (PEO) belongs to the group of the hydrophylic uncharged polymers used for the dynamic coating. PEO chains attached to surfaces are reported to reduce the protein and microbial adhesion [7,20,21]. The usage of a diluted PEO solution was demonstrated to increase the efficiency of the separation of the bacteria by CZE [1,14,22–26] or submicron-sized polystyrene particles. [27]. PEO has been also used in capillary electrophoresis as the non-bonded coating for modifying of EOF [28]. The presence of carrier ampholytes in the FS capillary and their adsorption onto the capillary wall mostly decreases EOF as well [15,29,30].

Generally, the capillaries for CIEF are usually preconditioned before each analysis by a mixture of carrier ampholytes [17,31–33]. Electroosmotic displacement of focused protein zones can be achieved by injection of the mixture of proteins and carrier ampholytes in form of a plug at the inlet of the capillary that has been pre-filled with the catholyte. The effect of the adsorption of carrier ampholytes onto uncoated FS capillaries is appreciable [29].

In isoelectric focusing, the resolution strongly depends on the slope of the pH gradient. When the resolution is not compromised by other factors of the process, the gradient manipulation is the main parameter used to improve the resolution [12,15]. The addition of the spacer ampholytes, β-alanine and 6-aminocaproic acid, improves the separation of haemoglobins in the pH gradient 6–8 in Ampholine gels [12,34]. In CIEF, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was added to the commercial carrier ampholytes Pharmalyte 3–10, in order to extend the separation range of pH gradient up to pH 12 [35]. A sandwich injection protocol [29] was used for the effective separation of amphoteric compounds having their p*I* values outside the pH range of the carrier ampholyte.

In this study we suggest the segmental injection of the sample mixture including the segment of spacers, the segment of bioanalytes and the segment of the carrier ampholytes with p*I* markers at the positive side of the inlet of the fused silica capillary that has been pre-filled with the catholyte. For the dynamic coating of the capillary low molecular weight poly(ethylene glycol), PEG 4000, is added to both end electrolytes, catholyte and anolyte, which prevents from the adsorption of bioanalytes and enables to control EOF. In order to obtain reproducible results we also suggest the washing of the capillaries between analyses by the mixture of organic solvents. For the tracing of the pH gradients the low-molecular p*I* markers [16] were used. The mixed cultures of microorganisms and proteins were reproducibly focused and separated by CIEF method suggested.

2. Materials and methods

2.1. Equipment

The capillary isoelectric focusing experiments were carried out using the laboratory-made apparatus [18]. All measurements were performed at the constant voltage (−) 20 kV supplied by the high voltage unit Spellman CZE 1000 R (Plainview, NY, USA). During the experiments, the current decreased from 40 to 60 μA at the beginning of experiment to 3–6 μA at the time of detection, depending on the sampling time interval and the sample solution. The lengths of the fused silica capillaries, 0.1 mm I.D. and 0.25 mm O.D. (Pliva-Lachema a.s., Brno, Czech Republic) were from 250 to 270 mm, with the effective lengths from 180 to 190 mm. The ends of the separation fused silica capillary were dipped in 3 ml-glass vials with the catholyte or the anolyte solutions (CaAn) and electrodes.

The suitable and reproducible linear velocities of focused zones and thus the regulation of EOF were achieved by help of the dynamic modification of the capillary inner surface by poly(ethylene glycol), $M_r = 4000$. The segmental injection of the sample was accomplished by siphoning action achieved by the elevating of the inlet – the anolyte reservoir, relative to the outlet – the catholyte reservoir [18]. The height difference of the reservoirs for the sample injection, Δh, can be adjusted in the range 100–180 mm for 7–60 s.

The on-column UV–vis detector LCD 2082 (Ecom, Prague, Czech Republic) connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, AZ, USA) was used at the wavelength 280 nm. The light absorption (optical density) of the microbial suspensions was measured using a DU series 520 UV–vis spectrophotometer (Beckmann Instruments, Palo Alto, CA, USA) at 550 nm.

The association between the cells was reversed by the sonication of their suspension in Sonorex, Bandelin electronic (Berlin, Germany). The frequency 35 kHz was used for one minute at the temperature 30 °C. After the sonication the microbial sample was vortexed for 10 min (Vortex-Genie 2, Scientific Industries, Bohemia, USA) and then immediately used.

The CZE experiments with EOF measurement were carried out using laboratory-made apparatus. All measurements were made at (−) 20 kV at constant conditions for the siphoning injection (100 mm, 5 s). The length of the whole separation fused silica capillary (0.1 mm I.D., 0.25 mm O.D.) was 320 mm with 250 mm to the on-column UV–vis detector.

The detector signals were acquired and processed with the Chromatography station for Windows CSW v. 1.5, DataApex s.r.o., Praha, Czech Republic; data were processed by graphic program Sigma-Plot 3.0, Jandel Scientific Software (Point Richmond, CA, USA).

2.2. Chemicals

The strains included in this study, *Saccharomyces cerevisiae* CCM 8191 (*S. cerevisiae*), *Escherichia coli* CCM 3954 (*E. coli*), *Candida albicans* CCM 8180 (*C. albicans*), *Candida parapsilosis* (*C. parapsilosis*), *Candida krusei* (*C. krusei*), *Staphylococcus aureus* (*St. aureus*), *Streptococcus agalactiae* CCM 6187 (*Str. agalactiae*), *Enterococcus faecalis* CCM 4224 (*E. faecalis*), *Staphylococcus epidermidis* CCM 4418 (*St. epidermidis*) and *Stenotrophomonas maltophilia* CCM 1640 (*S. maltophilia*) were obtained from Czech Collection of Microorganisms, Brno, Czech Republic. Bovine serum Albumin ($M_r = 67{,}000$, p*I* 4.9 [36]), Cytochrome *c*, horse heart ($M_r = 12{,}400$, p*I* 9.3 [36]), were from Sigma (St. Louis, MO,