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Microchip-based capillary electrophoresis of human serum proteins

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Abstract

The separation and relative quantitation of human serum proteins is important to the clinical diagnosis of various states of disease. Microchip-based capillary electrophoresis (CE) of human serum proteins offers several advantages over sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis and conventional CE methods, including decreased sample consumption and analysis time and the possibility of on-chip sample manipulation (dilution, labelling, etc.). The microchip used in these studies was designed to allow for on-chip, post-separation labelling of the proteins and subsequent laser-induced fluorescence detection. 2-Toluidinonaphthalene-6-sulfonate (TNS) is a virtually non-fluorescent reagent which, upon non-covalent association with the protein and excitation at 325 nm, produces a fluorescent product with an emission maximum near 450 nm. After optimization of buffer conditions (100 m*M* borate with 2 m*M* lactate, pH 10.5), individual serum proteins (IgG to mimic the γ zone, transferrin the β zone, α-1-antitrypsin the α_1 zone and albumin its own zone) were successfully resolved on-chip, as was a "synthetic" serum solution composed of a mixture of all four of the previously mentioned proteins. Analysis of all five protein zones in a true human serum sample, however, has not yet been achieved on-chip due to the poor sensitivity of the TNS label for several of the serum proteins. © 1997 Elsevier Science B.V.

Keywords: Derivatization, electrophoresis; Instrumentation; Proteins

1. Introduction

The separation and relative quantitation of human serum proteins has long been of importance to the clinical diagnosis of various states of disease. Although sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS–PAGE) in slab format has traditionally been employed for serum separations, it has recently been demonstrated that free solution capillary electrophoresis (CE) provides a viable alternative [1–6]. Advantages offered by CE in such a clinical application include decreased sample consumption, decreased separation time and increased resolution. Transferring this clinical method to the microchip environment offers further advantages. Amongst these are further reductions in sample consumption and analysis time, and the possibility of on-chip sample manipulation (such as dilution or labelling) and multi-channel capacity.

CE determination of proteins often employs UV absorbance detection. However, in the microchip environment, extremely short path lengths result in low absorbance values, so the more sensitive laser-induced fluorescence (LIF) detection is commonly used. Fluorescent labelling of proteins is best accomplished after their separation and immediately before detection to avoid problems with multiple site labelling. The microchip design chosen for these studies provided on-chip, post-separation labelling of the proteins with 2-toluidinonaphthalene-6-sulfonate

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(TNS) and subsequent LIF detection. Optimal separation conditions were then determined to allow for separation, labelling and detection of four proteins in a "synthetic serum" sample in less than 60 s on-chip .

2. Experimental

2.1. Solutions and reagents

In most instances, protein separations were conducted with a 100 m*M* borate buffer prepared by dissolving boric acid (J.T. Baker, Phillipsburg, NJ, USA) in doubly-distilled, deionized water along with 2 m*M* calcium lactate (Sigma, St. Louis, MO, USA). The addition of lactate as a "stabilizer" to the borate buffer was recommended by Bio-Rad (Hercules, CA, USA) [7]. The pH was adjusted to 10.5 by the addition of a 1.0 *M* solution of NaOH (BDH, Toronto, Canada). Buffer pH and concentration were varied to determine their effect on the resolution of proteins.

A 50 m*M* tricine buffer was also used in some studies. This buffer consisted of 50 m*M* tricine (Sigma), approximately 28 m*M* NaCl (Sigma) and 0.01% (v/v) Tween 20 (Aldrich, Milwaukee, WI, USA) and the pH was adjusted to 8.0 by the addition of 1.0 *M* NaOH. Tricine buffer was prepared fresh every other day.

The labelling reagent was prepared by dissolving 2-toluidinonaphthalene-6-sulfonate (TNS) (Sigma) to a concentration of 0.2 m*M* in the borate buffer described previously. Additionally, some preliminary labelling studies involving 0.2 m*M* 1-anilinonaphthalene-8-sulfonate (ANS) (Sigma) and 3.88 m*M* *o*-phthaldialdehyde (OPA) (Aldrich) solutions prepared in borate buffer were conducted. The OPA labelling solution was prepared by dissolving OPA in ethanol (Fisher Scientific, Nepean, Canada) and mercaptoethanol (Aldrich) and diluting with borate buffer. Final ethanol and mercaptoethanol concentrations were 2% (v/v) and 0.05% (v/v), respectively. Buffer and labelling reagent solutions were passed through a cellulose acetate syringe filter (0.2 μm pore size; Nalge, Rochester, NY, USA) prior to use.

Human serum was obtained from Bio-Rad and individual human serum proteins (IgG, α-1-antitrypsin, transferrin and albumin) from Sigma. Protein solutions were prepared just before use by dilution in borate buffer and filtration through 0.45 μm cellulose acetate syringe filters (Lida, Kenosha, WI, USA).

2.2. Post-separation labelling: chip design

Microchip devices were prepared in either pyrex glass (Paragon Optical, Reading, PA, USA) or synthetic quartz (Hoya, San Jose, CA, USA) by the Alberta Microelectronics Centre (Edmonton, Canada). Sample injection, separation and labelling channels were etched to a depth of 14 μm in the 2 mm thick pyrex or quartz glass according to a previously-described micromachining technique [8]. Thermal bonding of cover plates, predrilled for access to the etched channels, served to complete the CE chips, and was carried out as described elsewhere [8].

The channel structure used for post-separation labelling is illustrated in Fig. 1. Exact dimensions are given in Ref. [8]. This structure employs a double-T injector for sample introduction [9], and a separation channel approximately 49 mm in length. This length

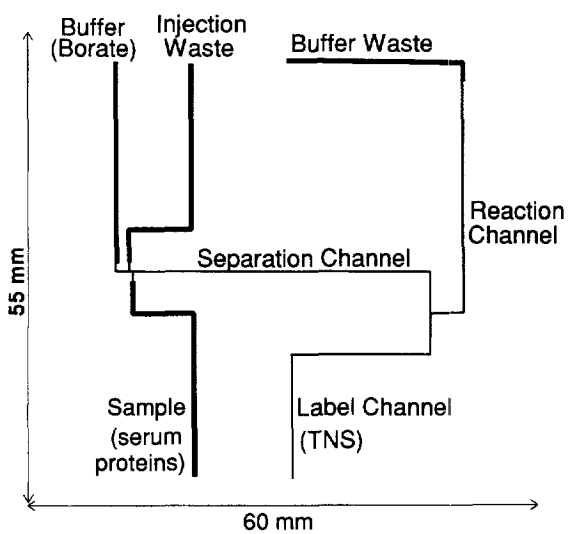

Fig. 1. Schematic diagram of the post-separation labelling design of the chip. Thick lines were 256 μm wide, thin lines were 66 μm wide. Channels were 14 μm deep. The indicated channel lengths are only approximately to scale.

is sufficient to ensure that sample components are well resolved prior to their mixing with labelling reagent which is introduced at a T-shaped junction (see Fig. 2). Detection optics are positioned just downstream from the T-junction, not more than 10 mm from where the labelling reagent and separated sample zones first contact each other. In this way, we are able to maintain the integrity of the separated sample zones even after labelling. Other post-separation labelling structures have been tested and shown to provide superior mixing [8], however, in these studies, detection was limited not by mixing but by the affinity of the labelling reagent for certain proteins.

Chips were conditioned for use by filling first with water and then subsequently flushing with 1.0 *M* NaOH, water and borate buffer. Before storage overnight or for extended periods of time, the chips were flushed with 1.0 *M* NaOH and water, and were left filled with water. Sample, buffer and waste reservoirs on the chip were simply constructed from pipette tips which were cut to size and inserted into the pre-drilled holes of the chip's cover plate.

2.3. Instrumentation

The power supply and relay system used to control the electrophoretic voltages necessary for sample injection, separation and labelling in microchip CE experiments has been described previously [8]. Computer control of the voltage system and of data acquisition was achieved by way of LabView programs (National Instruments, Austin, TX, USA) written in-laboratory. During injection, the sample and injection waste reservoirs were held at ground and −1.5 to −1.8 kV, respectively. Subsequently, separation was achieved by applying −8.0 kV at the buffer waste reservoir with the buffer reservoir grounded. Simultaneously, the label reservoir was held at −3.6 kV to provide adequate flow of labelling reagent into the separation stream.

The LIF detection system in these studies, which employed a 325 nm, 9.3 mW He–Cd laser (Omnichrome, Chino, CA, USA), is the same as that already described by this group [8], except that a 450 nm (±10 nm) optical band pass filter (Omega Optical, Brattleboro, VT, USA) was used.

Conventional CE experiments were conducted using a Waters Quanta 4000 CE instrument (Bradford, MA, USA) with pressure injection and UV absorbance detection at 214 nm. Absorbance data were recorded by a strip chart recorder. Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used with the following nominal dimensions: 52.80–55.50 cm total length 45.10–48.00 cm effective length (inlet to detector), 51 μm inside diameter and 355 μm outside diameter. Capillary conditioning consisted of sequentially flushing the capillary (by pressure) with 1 *M* NaOH for 10 min, with water for 10 min and with buffer for 30 min at the beginning of each day. The capillary was not rinsed between runs and was stored filled with doubly-distilled, deionized water when not in use. All experiments were conducted at ambient temperature (25°C).

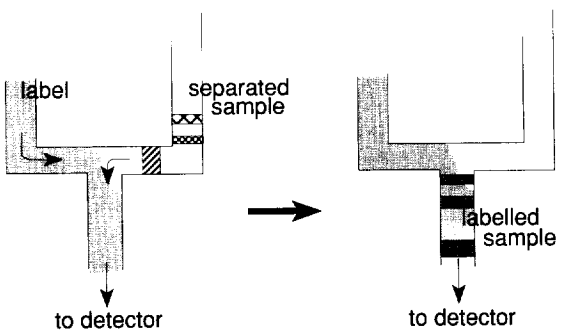

Fig. 2. Labelling reagent is driven by electroosmotic flow to a mixing-T intersection, where it meets and mixes with separated sample zones. Labelled sample zones are then detected just downstream from the mixing point.

3. Results and discussion

3.1. Optimization of serum protein separation conditions

A variety of buffer systems have been successfully employed for the separation of serum proteins [2]. In these studies, the suitability of two buffers – tricine and borate – was determined by preliminary experiments using a Waters Quanta 4000 CE instrument. A 50 m*M* tricine buffer (pH 8.0) gave significantly poorer resolution of serum proteins than did borate buffers, and so was not studied in further detail.

Resolution of the γ, β, α_2, α_1 and albumin zones of human serum was readily achieved in a 75 m*M* borate buffer (pH 10.0) with 1 m*M* lactate, as demonstrated in Fig. 3. It was found that both 1 m*M* and 2 m*M* lactate concentrations gave the same quality CE separations. However, the concentration and pH of the borate buffer were varied in order to determine the conditions leading to the greatest separation of serum proteins. This optimization was necessary since the method was to be transposed to the microchip environment, where we used lower voltages than are commonly used in conventional CE.

Buffer pH can be used to alter the charge on both the capillary wall and the proteins, thereby affecting the electroosmotic flow-rate and protein mobility, as well as the extent of protein adsorption on the capillary wall. Fig. 4 shows the change in migration time of two serum proteins – IgG and α-1-antitrypsin – as a function of buffer pH. At pH 10.5 and above, the difference in migration time between IgG and α-1-antitrypsin increased dramatically. Fig. 4 shows that pH 10.5 is sufficient to provide the increased resolution required for a successful separation on-chip. Additionally, at this pH, the serum proteins are negatively charged, and so should be electrostatically repelled from the negatively-charged capillary wall. Such repulsion should minimize protein adsorption and its consequent band broadening. Although buffer pH values greater than 10.5 may offer similar protein resolution and further reductions in protein–wall interactions, such pH extremes may result in less reproducible electroosmotic flow and in reduced fluorescence emission of the protein–TNS complex [10].

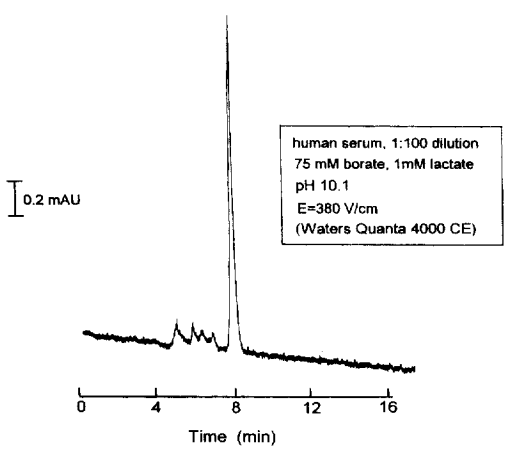

Fig. 3. Electropherogram (obtained by conventional CE) of diluted human serum. Capillary dimensions: 52.80 cm (45.10 cm effective length)×51 μm I.D.×355 μm O.D.. Injection by pressure for 12 s. Separation voltage: 20.00 kV. Detection by UV absorbance at 214 nm. Buffer and sample conditions as stated in the Figure.

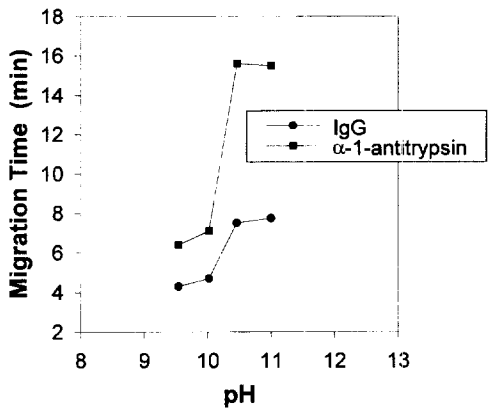

Fig. 4. The effect of borate (75 m*M*) buffer pH on migration time of human IgG and α-1-antitrypsin.

The effect of the concentration of the borate buffer on serum protein separation was also determined. Typically, increasing the concentration, or ionic strength, of the separation buffer leads to a decrease in electroosmotic mobility. A decrease in electroosmotic mobility should increase the opportunity for complete resolution of the proteins by providing more time for the separation to occur. This was observed experimentally. When the concentration of borate (pH 10.5) was increased from 75 m*M* to 100 m*M*, the electroosmotic mobility decreased from $5.1 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} to $4.0 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. Concomitantly, the difference in migration times between α-1-antitrypsin and IgG increased from 8.1 min to 10.9 min. Further increasing the buffer concentration to 150 m*M* borate resulted in unstable baselines and excessively long migration times, and so was not a suitable alternative.

3.2. On-chip, post-separation labelling of human serum proteins

Conducting human serum protein separations on-chip required not only the transfer of the optimized

buffer conditions (100 m*M* borate with 2 m*M* lactate, pH 10.5), but also the development of a post-separation labelling scheme. By labelling after separation rather than before, the distorting influence of multiple labelling is minimized and even weakly associated, non-covalent dye–protein complexes can be detected. Post-separation labelling of proteins requires the interaction between protein and dye molecules be both complete and fast. This is necessary since detection of the labelled species occurs just downstream of the dye–protein mixing point. If detection occurred further from the mixing point, then separation or partial separation of multiply-labelled species could occur, resulting in band broadening or multiple peak formation.

Several labelling reagents were studied for their suitability in this work. TNS is a weakly fluorescent species which, upon intercalation into the hydrophobic regions of proteins, undergoes a dramatic increase in fluorescence quantum efficiency [10]. Fluorimetry studies (Shimadzu RF-5000 spectrofluorophotometer) showed that serum protein–TNS complexes were very fast to form and were long-lived, and that excitation and emission maxima were located near 325 and 440 nm, respectively. Similar studies with ANS as a label for serum and IgG samples showed higher background and lower signal. OPA is a common label for amino acids, but OPA–protein complexes were not successfully resolved on-chip in these studies. Consequently, 0.2 m*M* TNS solutions, prepared in borate buffer, were used in these experiments. According to Swaile and Sepaniak [10], increasing the concentration of TNS beyond 0.2 m*M* results not only in an increase in fluorescence signal, but also a marked increase in background fluorescence and baseline noise. Additionally, it should be noted that the fluorescence of the TNS–protein complex is pH dependent, with signal intensity decreasing as pH increases [10]. However, the buffer pH in these experiments was chosen on the basis of protein resolution considerations, and so some fluorescence intensity was necessarily sacrificed.

To demonstrate the feasibility of on-chip, post-separation labelling of human serum proteins with TNS, a "synthetic serum" sample was prepared in borate buffer. The first sample contained 2.5 mg/ml human IgG, 2.5 mg/ml transferrin and 0.6 mg/ml α-1-antitrypsin, to represent the γ, β and α_1 zones, respectively. These proteins were separated, labelled with TNS, and detected on-chip, as illustrated in the bottom trace of Fig. 5. (The α-1-antitrypsin component contributes two peaks to the electropherogram, the second and largest of which we believe is an albumin impurity. The α-1-antitrypsin samples were also run using the conventional CE with absorbance detection. The two peaks observed matched the migration times of the α_1 and albumin peaks found in serum samples.) Addition of 0.8 mg/ml albumin to the synthetic serum mixture resulted in the electropherogram shown in the top trace of Fig. 5. The correspondence of the albumin peak and the α-1-antitrypsin impurity peak further confirms the identity of the impurity as human serum albumin. It is evident that the chip can deliver sufficient resolving power to separate and label four of the principal human serum proteins on-chip in less than 60 s. This performance compares favourably with the conventional CE separation of human serum proteins shown in Fig. 3.

The fluorescent labelling reaction with TNS is not very effective with real serum samples, since TNS preferentially associates with albumin rather than with globulin-type proteins [11]. A solution prepared

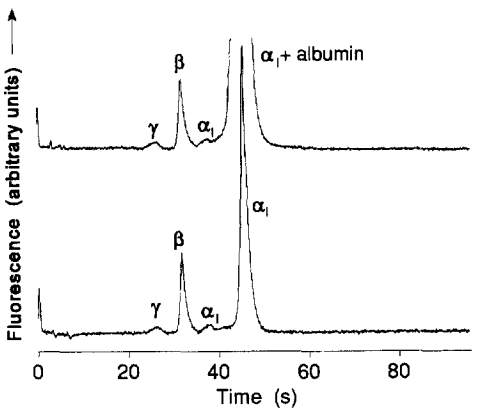

Fig. 5. Electropherograms of human serum proteins obtained by microchip CE with post-separation labelling and LIF detection. Synthetic serum sample contained 2.5 mg/ml human IgG, 2.5 mg/ml transferrin and 0.6 mg/ml α-1-antitrypsin (lower electropherogram) plus 0.8 mg/ml albumin (upper electropherogram) prepared in 100 m*M* borate buffer (pH 10.5) with 2 m*M* lactate. Labelling reagent: 0.2 m*M* TNS in borate buffer. Electrokinetic injection for 10 s at 1.8 kV. Separation voltage: 8.0 kV.

at the physiological concentration of albumin gave a 125-fold larger fluorescence signal than did a solution of human IgG prepared at its physiological concentration. Due to leakage effects from the sample channel of the chip, a background level of 1–3% of serum can be expected in the separation channel [12,13]. Consequently, this did not allow the required dynamic range to conduct measurements at normal physiological serum protein concentrations. For this reason, the synthetic samples were prepared to show that the required resolution of the various proteins was attainable. Nevertheless, we measured real human serum samples diluted fifty-fold in borate buffer. The electropherograms showed a single peak, corresponding to the albumin zone of serum. Less than 1% relative standard deviation in the migration time of the albumin peak was achieved, with an average number of theoretical plates of 8700 [±400 (standard deviation)]. Working with serum samples any more concentrated than a 10-fold dilution resulted in adsorption problems with the channel walls and so was not feasible.

4. Conclusions

Microchip-based CE provides a method with integrated separation and labelling capabilities for human serum proteins. A simple 100 m*M* borate buffer with 2 m*M* lactate and pH adjusted to 10.5 was found to be optimum for these serum protein separations based on studies of the effects of pH and concentration on protein migration. Proteins were first separated, and then still within the chip channel structure, were mixed with 0.2 m*M* TNS labelling reagent, also prepared in borate buffer. Just downstream from the mixing point, labelled proteins were detected by way of LIF. In this manner, a mixture of four human serum proteins were resolved in less than 60 s. This compares favourably to a conventional CE separation of a diluted serum sample, which required nearly 8 min to resolve the five major protein zones. On-chip separation of true human serum samples, however, has not yet been achieved due to differential complexation of the chosen fluorescent label (TNS) with serum proteins. However, the resolving power of the microchip CE system appears to be more than sufficient to separate true serum samples. This microchip CE method thus appears to be limited only by the effective detection of the separated serum proteins. The use of alternative fluorescent labels or of the on-chip UV absorbance detection cell currently being developed in this lab are promising, and the ability to fabricate a wide range of channel structures could lead to a multi-channel serum protein device with true clinical capabilities. Furthermore, the possible use of plastic, disposable microchip devices [14] in this application has obvious advantages for clinical laboratories.

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