Chiral separations using an immobilized protein-dextran polymer network in affinity capillary electrophoresis

Peng Sun, Geoffrey E. Barker and Richard A. Hartwick*

Department of Chemistry, State University of New York at Binghamton, Binghamton, NY 13902 (USA)

Nelu Grinberg

Merck Research Laboratories, Rahway, NJ (USA)

Roman Kaliszan

Medical Academy, Gdansk (Poland)

ABSTRACT

An improvement in affinity capillary electrophoresis (ACE) was achieved by covalently linking bovine serum albumin (BSA) to a high-molecular-mass dextran (M_r 2 000 000) using cyanogen bromide. The efficiency of the binding reaction was greater than 99%, measured through quantitative separation of the protein and protein–dextran polymer network mixture on a bare capillary using capillary electrophoresis (CE). Baseline separation of leucovorin (LV) enantiomers was obtained under 9 min using a linear polyacrylamide-coated capillary (with an effective length of 20 cm) filled with the BSA–dextran polymer network. The present work offers several significant advantages over other approaches to ligand immobilization, and can be generally applied to a wide variety of ligand–substrate systems.

INTRODUCTION

With the improved understanding of the biological action of drugs with respect to their stereochemistry, investigations concerning the pharmacology and toxicology of individual drug enantiomers have become increasingly more important. Therefore chiral separation of pharmaceutical compounds is currently of great concern. Many liquid chromatographic techniques for the separation of enantiomers have been developed and chiral stationary phases are now widely available commercially. Among the

various chiral stationary phases investigated, immobilized protein phases such as α-acid glycoprotein (AGP) [1,2], ovomucoid [3–5], and bovine serum albumin (BSA) [6–8] have been quite successful in a variety of separations, although separation efficiencies have often been poor [9]. Columns are also quite expensive, and have limited lifetimes.

CE is a technique which has developed considerably during the past several years [10–12]. CE distinguishes itself from other liquid-phase separation methods in that high efficiencies (usually greater than 100 000 plates) are obtained. Furthermore, CE can be viewed as a micro-scale to nano-scale analytical procedure and is advantageous when the availability of sample, mobile

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* Corresponding author.

phase, or separation phase is limited. Separation of racemic mixtures using CE has been reported so far using cyclodextrin (CD) [13,14], ligand-exchange principles [15,16], and mixed micelles in the run buffer [17,18]. Recently, a method for chiral separation of leucovorin (LV) using BSA as a run buffer additive was developed in our laboratory [19].

To date, capillary affinity gel electrophoresis (CAGE) has developed rapidly. It has opened up new horizons for the biospecific separation of DNA and RNA as well as investigating the interactions between biological molecules. Using capillaries filled with conjugated polyacrylamide-poly(9-vinyladenine) gel, Baba *et al.* [20] achieved the base-specific separation of oligo-deoxynucleotides. A CAGE method for the separation of tryptophan enantiomers using capillaries filled with gel consisting of BSA cross-linked with glutaraldehyde was recently reported by Birnbaum and Nilsson [21]. Baseline separation of tryptophan enantiomers was obtained by this method. However, a limitation of this method is detection interference caused by the opaque off-white color of the BSA cross-linked gel. To overcome this problem, the capillary must be partially filled with the gel, with detection of sample occurring at a point on the capillary that is not occupied by the gel. This creates fabrication difficulties, and in addition, once the gel is formed in the capillary it is permanent and cannot be removed.

In this paper, a method is presented for synthesizing BSA–dextran conjugates. The co-valent linking of a protein, DNA or other species to a high-molecular-mass, UV-transparent dextran polymer network [22] offers several significant advantages over existing methods. Since the network is replaceable, fresh stationary phase can be used after each injection if necessary, with good reproducibility. The phase ratio of the ligand site can be controlled by mixing ligand–polymer with plain polymer in any proportion. Polymer networks are also less subject to channeling and other defects arising when the capillaries are coiled or moved. The feasibility of using a replaceable BSA–dextran polymer network for chiral separations is presented in this paper.

EXPERIMENTAL

Chemicals

Acrylamide, N,N,N',N'-tetramethylethylene-diamine (TEMED), and ammonium persulfate (APS) were obtained from Bio-Rad Labs. (Richmond, CA, USA). Dextran (M_r 2 000 000), cyanogen bromide, and BSA were purchased from Sigma (St. Louis, MO, USA). 3-Meth-acryloxypropyltrimethoxysiline was obtained from HULS America (Bristol, PA, USA) and calcium leucovorin (LV) from Lederle Laboratories, American Cyanamid (Pearl River, NY, USA). All other chemicals were from Fisher Scientific (Fair Lawn, NJ, USA).

Apparatus

A CE apparatus which was designed and constructed in our laboratory was used. It contains a Spectra 100 UV detector (Spectra Physics, Reno, NV, USA), a CZE 1000 PN 30 high-power voltage supply (Spellman, Plainview, NY, USA), and a high-power supply local controller (Chamonix Industries, Binghamton, NY, USA). The controller box could be programmed for electrokinetic sample introduction as well as adjustment of the actual separation voltage. The electropherograms were processed on SP 4400 integrator (Spectra Physics).

Polymer network synthesis and preparation of capillaries

The immobilization of BSA on dextran was accomplished by the method reported by Taka-hura *et al.* [23] with some modification (scheme of synthesis reaction is shown in Fig. 1). Dextran (200 mg) was dissolved in 10 ml of 10 m*M* phosphate buffer at pH 7.12. This solution was degassed for 20 min, then it was activated by cyanogen bromide (20 mg) at pH 10.7 for 20 min [24]. Afterwards the pH was adjusted to 9.0 and BSA (20 mg) was added to this solution. The binding reaction was allowed to proceed for 12 h at room temperature. The efficiency of this binding reaction was determined by CE using a bare fused-silica capillary (75 μm I.D. × 360 μm O.D., Polymicro Technologies, Phoenix, AZ, USA).
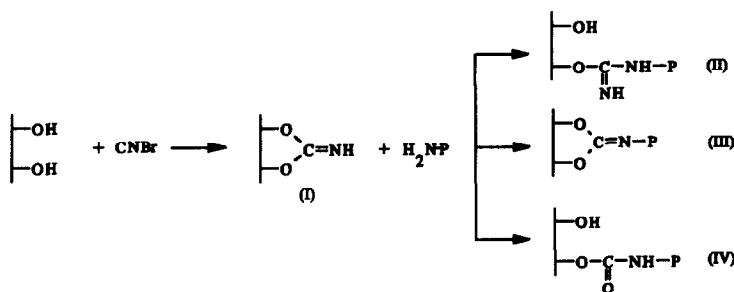
Fig. 1. Scheme of immobilization of BSA on dextran. I = Active imidocarbonate; II = N-substituted isoureas; III = N-substituted imido carbonate; IV = N-substituted carbamates.

Capillaries were coated with linear polyacrylamide by the method reported by Hjertén [25]. After being rinsed with water, the coated capillary was filled with the BSA–dextran network by flushing the capillary with the polymer network solution. The capillary was equilibrated by applying an electric field of 200 V/cm for 20 min using a run buffer consisting of 10 mM phosphate at pH 7.12. The samples, e.g. LV, were injected through electrokinetic injection. The BSA–dextran polymer network can be removed and replaced by means of a syringe.

RESULTS AND DISCUSSION

Immobilization of BSA on dextran

Complete reaction of the ligand to the polymer network is essential for good separation efficiency, otherwise the unbound ligand will travel at a velocity different from the bound fraction, creating the equivalent of active sites on a standard chromatographic separation. The efficiency of binding reaction of BSA on dextran was determined by CE to be greater than 99%. Fig. 2 shows an electropherogram of the BSA–dextran polymer network sample. Only a single peak is detectable at 5.65 min, which was identified as the BSA–dextran polymer network peak. No detectable peak was observed at the migration time for free BSA (7.7 min). The small shoulder on the front of the BSA–dextran peak is thought to be due to MW distributions within the dextran polymer, although it could also arise from different bonding densities of BSA with the dextran, with resultant small mobility differences.
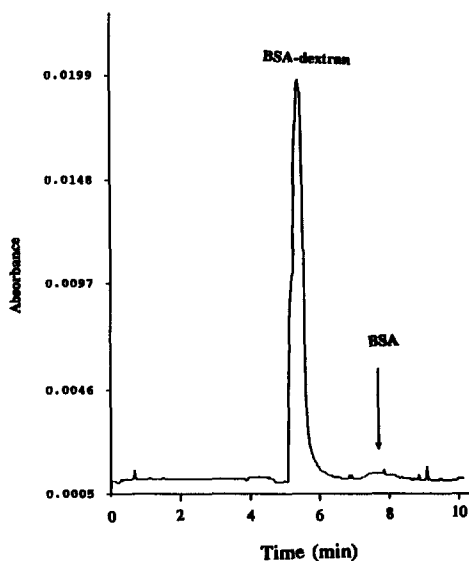
The mobility of the BSA–dextran complex was estimated to be ca. $3 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1}. It is interesting to note that this mobility results in a migration time of ca. 17 h for the BSA–dextran complex to travel one column length, in the absence of any electroosmotic flow. The capillaries used in this work are coated with linear polyacrylamide, and electroosmotic flow of the coated capillaries was found to be very slow (neutral molecules did not migrate to the detection window in several hours when 300 V/cm high voltage was applied). In many situations, a

Fig. 2. Electropherogram of BSA–dextran polymer network sample. Capillary: fused silica (75 μm I.D. × 360 μm O.D.), 35.3 cm effective length. Voltage: 280 V/cm. Buffer: 20 mM phosphate at pH 7.4.

small electroosmotic flow will exist, usually opposite in direction to that of the protein complex. This results in a very stable system, in which the polymer will not flow out of the capillary to any significant extent during the course of several hours of running.

Chiral separation of leucovorin (LV) by BSA–dextran polymer network filled capillary

Baseline separation of the LV enantiomers could be obtained in just under 9 min on a BSA–dextran polymer network filled capillary with an effective length of 20 cm (Fig. 3). The short analysis time could not be achieved when using free BSA as the buffer additive [19] since in that method BSA moves in the same direction as the sample, creating a need for a relatively long effective capillary length to achieve adequate resolution. The stability of the BSA–dextran polymer network filled capillary was evaluated using the LV sample. No significant loss in efficiency was observed after 20 injections over the course of ca. 5 h. Replenishment of the capillary with fresh polymer when required is simple, and takes only minutes. The fresh BSA-polymer has the advantage of offering a virgin stationary phase for the solutes, such that if dirty samples are injected, capillaries can be recreated between injections if necessary.

The 6R isomer interacted more strongly with the immobilized BSA than did the 6S isomer, and also exhibited considerable peak distortion
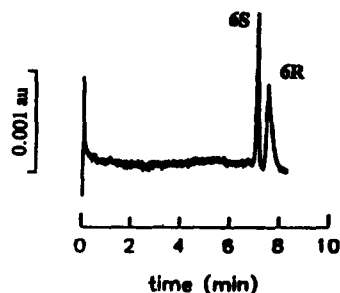
in comparison to the 6S species. Similar effects were observed in previous free solution separations of LV with BSA [19]. It is not known at this time whether the differences in peak width arise from differences in steric hindrance of the isomers for two distinct sites on (or within) the protein, or if the peak widths reflect differences in the adsorption energetics and kinetics of both isomers to the same site(s).

The advantage of covalent binding of a protein ligand to the polymer network is that the phase ratio of the ligand can be readily varied by dilution with non-derivatized dextran. This can be useful for solutes which absorb too strongly to the ligand for facile elution. The effect of phase dilution was studied for the BSA-LV system, and are presented in Fig. 4. Fig. 4A–D show the effect of diluting a 2 mg/mL BSA-dextran network with dextran. The resolution decreases systematically with decreasing phase ratio. The graph shown in Fig. 5 plots the trend. Both the resolution and the absolute migration times of both the 6R and 6S isomers decrease with decreasing phase ratio. This supports earlier conclusions that both isomers are retained to some extent by the BSA molecule [19], with the
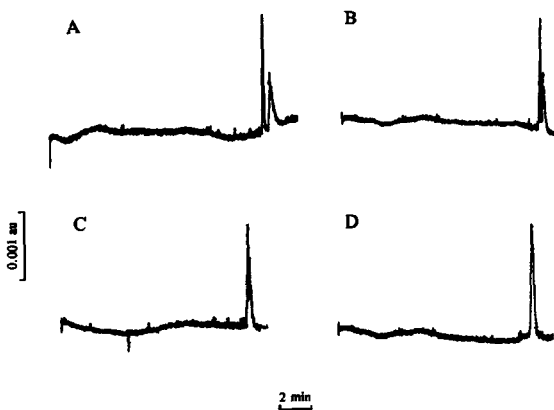
Fig. 3. Separation of leucovorin (LV) enantiomers on a capillary filled with BSA–dextran polymer network. Conditions: linear polyacrylamide-coated capillary with 20 cm effective length; electric field strength, 217 V/cm; pH 7.02, 20 mM phosphate buffer; injection, 5 kV × 5 s; sample, 0.1 mg/ml leukovorin (LV) in buffer; detection wavelength 230 nm.

Fig. 4. Electropherograms showing the effect of diluting a 2 mg/ml BSA–dextran network with dextran solution. Conditions are the same as those of Fig. 3 except electric field strength is 250 V/cm and the effective length of the capillary is 40 cm. Sample: 0.5 mg/ml leukovorin (LV) in buffer. Injection: 5 kV × 4 s. BSA concentration: A = 2 mg/ml; B = 1 mg/ml; C = 0.5 mg/ml; D = 0.25 mg/ml. Time axes start at 0 min and chart speed is 0.5 cm/min.
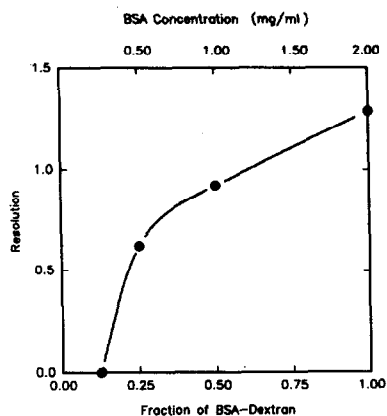
Fig. 5. Effect of BSA-dextran polymer network concentration on the resolution of leucovorin.

6R isomer being significantly more strongly retained than the 6S. It is possible that both isomers are retained by non-specific adsorption to BSA, with only the 6R isomer interacting with a particular site, or it is possible that both solutes are interacting with a chiral site, but to different degrees. Work is in progress in our laboratory to further elucidate the exact retention mechanisms operating in this LV-BSA system.

In conclusion, the authors have shown the feasibility of employing an immobilized protein-dextran polymer network for chiral separation via affinity capillary electrophoresis. Several significant technical advantages are created by using a covalently immobilized protein-polymer network over existing methodologies. Entrapment of BSA using gels cross-linked with glutaraldehyde (physical entrapment) [21] are not UV transparent, such that detection must be confined to a physically defined detection region. The polymer network used is UV transparent, eliminating this problem. In common with other polymer network methods, the lifetime of the capillary is extended by the ability to clear and refill the capillary at will with fresh polymer. For strongly retained solutes, resolution and retention times can be controlled by diluting the bound-polymer, thus controlling the phase ratio.

While data was presented only for a single test separation, that of leucovorin interacting with BSA, it is obvious that this is a completely general system. Not only can innumerable protein-solute interactions be used, often with rare and expensive proteins, but DNA-protein and DNA-DNA interactions can also be readily studied. A particularly exciting area of application is to the study of drug structure libraries. It should be possible to readily study drug-solute interactions, with the goal of screening structures for potential activity, at considerable savings in reduced animal studies. Another area of study is the competitive binding of different drugs to carrier proteins, such as the albumins or glycoproteins, which will yield useful in vitro information on potential cross-reactivity problems during dosing. Furthermore, in the area of chiral separations alone, other solutes, such as cyclodextrins, 3-point ligands and metal chelates can be used to advantage with polymer networks to create a convenient "stationary phase" for electrically driven separations.

ACKNOWLEDGEMENTS

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