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Short communication

Effective electrophoretic mobilities and charges of anti-VEGF proteins determined by capillary zone electrophoresis

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ABSTRACT

Macromolecules such as therapeutic proteins currently serve an important role in the treatment of eye diseases such as wet age-related macular degeneration and diabetic retinopathy. Particularly, bevacizumab and ranibizumab have been shown to be effective in the treatment of these diseases. Iontophoresis can be employed to enhance ocular delivery of these macromolecules, but the lack of information on the properties of these macromolecules has hindered its development. The objectives of the present study were to determine the effective electrophoretic mobilities and charges of bevacizumab, ranibizumab, and model compound polystyrene sulfonate (PSS) using capillary zone electrophoresis. Salicylate, lidocaine, and bovine serum albumin (BSA), which have known electrophoretic mobilities in the literature, were also studied to validate the present technique. The hydrodynamic radii and diffusion coefficients of BSA, bevacizumab, ranibizumab, and PSS were measured by dynamic light scattering. The effective charges were calculated using the Einstein relation between diffusion coefficient and electrophoretic mobility and the Henry equation. The results show that bevacizumab and ranibizumab have low electrophoretic mobilities and are net negatively charged in phosphate buffered saline (PBS) of pH 7.4 and 0.16 M ionic strength. PSS has high negative charge but the electrophoretic mobility in PBS is lower than that expected from the polymer structure. The present study demonstrated that capillary electrophoresis could be used to characterize the mobility and charge properties of drug candidates in the development of iontophoretic drug delivery.

1. Introduction

Antibodies have been studied for disease specific target therapies through specific binding to the target [1]. In addition, antibodies can be an effective targeting moiety for the conjugation with small molecule drugs to increase the site specificity and therapeutic window of the drugs [2]. A number of antibody therapies and delivery systems are either approved or in clinical development. Among the different classes of antibodies shown to be effective, anti-human vascular endothelial growth factor (anti-VEGF) antibody bevacizumab (Avastin) and antibody fragment (Fab) ranibizumab (Lucentis) have been used in the treatment of posterior eye diseases such as age-related macular degeneration, choroidal neovascularization, diabetic retinopathy, and macular edema. Bevacizumab is the first anti-angiogenic humanized recombinant monoclonal antibody approved by FDA for metastatic cancers. As an anti-VEGF protein, intravitreal injection of bevacizumab was shown to be beneficial in off-label treatment of neovascular eye diseases [3,4]. Ranibizumab is a monoclonal Fab fragment from the same parent antibody as bevacizumab. It is an FDA approved agent shown to be effective in the treatment of wet age-related macular degeneration and diabetic retinopathy.

Iontophoresis is a method to enhance the delivery of a compound across a biomembrane with the assistance of an electric field. During iontophoretic drug delivery, a low electric potential is applied to drive a drug into and across a tissue via the mechanisms of electrophoresis (direct electric field effect), electroosmosis (electric field induced convective solvent flow), and electro-permeabilization (electroporation) [5–7]. Iontophoresis has been successfully employed in drug administration across the skin for local and systemic drug delivery. Ocular iontophoresis has also been studied for its utility in noninvasive drug delivery to the eye [8,9]. Recently, a number of ocular iontophoresis studies have demonstrated the effectiveness of ocular iontophoretic delivery and its safety [10,11]. However, the mechanisms of transscleral iontophoretic delivery of bevacizumab and ranibizumab such as the interplay of electrophoresis and electroosmosis in iontophoretic transport are not fully understood [12], partly due to the lack of information on the effective electrophoretic mobilities and charges of these agents. Effective transscleral iontophoretic delivery of these macromolecules requires the understanding of these two

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mechanisms and their relative contributions in iontophoretic transport.

Capillary electrophoresis is an analytical method that can be used to assay a diverse array of analytes such as biologics and pharmaceuticals [13–15]. Capillary zone electrophoresis is the simplest form of capillary electrophoresis and utilizes an open capillary column connected to two buffered reservoirs. Capillary electrophoresis was previously employed to study the physicochemical properties such as electrophoretic mobilities and structures of proteins [16,17], natural organic matter [18], and oligonucleotides [19]. It was also used in the determination of dissociation constants [20,21] and octanol/water partition coefficients [22] of pharmaceuticals. In drug delivery, the utility of capillary electrophoresis in characterizing the electric properties of drugs and predicting transdermal iontophoretic delivery was also demonstrated [19,23,24].

The objectives of the present study were to (a) determine the intrinsic electrophoretic mobilities of bevacizumab, ranibizumab, and a model polyelectrolyte polystyrene sulfonate using capillary zone electrophoresis and (b) calculate the effective charges of these macromolecules. This information will be particularly useful in drug delivery method development such as ocular iontophoresis. The electrophoretic mobilities and molecular charges of salicylate, lidocaine, and BSA were also determined and served as the anion, cation, and macromolecule controls, respectively, to validate the methodology in the present study.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS, pH 7.4, consisting of 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride) was prepared by dissolving PBS tablets (Sigma–Aldrich, St. Louis, MO) in distilled, deionized water. PBS of 0.016 and 0.04 M ionic strength was prepared by diluting PBS to the appropriate ionic strength with distilled, deionized water. Millipore filters (Nylon, Zymark®, 0.45 μm pore diameter) were purchased from Millipore Corp. (Bedford, MA). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO). Poly(styrene sulfonic acid) sodium salt (PSS, MW 67 kDa, Mw/Mn < 1.2) was purchased from Polysciences, Inc. (Warrington, PA). Bevacizumab (Avastin®, 100 mg in 4 mL) and ranibizumab (Lucentis®, 2 mg in 0.2 mL) were from Genentech, Inc. (Oceanside, CA). Salicylic acid (sodium salt), lidocaine (hydrochloride salt), and benzyl alcohol were from Sigma–Aldrich (St. Louis, MO). Benzyl alcohol, salicylate, lidocaine, BSA, PSS, bevacizumab, and ranibizumab solutions at concentrations ranging from 0.01 to 0.2% (w/w) were prepared in PBS.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed using a Beckman P/ACE MDQ analytical capillary electrophoresis system (Beckman Coulter, Brea, CA) equipped with a diode array detector. The columns were uncoated bare fused silica capillary columns, 30 cm total length and 20 cm length to detector window, and 50 μm I.D. (BIOTAQ Inc., Washington DC) and 75 μm I.D. (Agilent Technologies, Santa Clara CA). The analytes of interest, bevacizumab and ranibizumab, were evaluated in both columns and the results were combined in the analyses. In the experiments, the capillaries were pretreated with 0.1 M NaOH rinse for 0.5 or 1 min, followed by rinsing with deionized water for 0.5 or 1 min, and then the background electrolyte solution for 1 or 2 min, initially and between each run, all at a pressure of 20 psi, for the 75 and 50 μm I.D. columns, respectively. PBS of 0.016, 0.04, and 0.16 M ionic strength was the background electrolyte solutions. The condition of 0.16 M ionic strength and pH 7.4 was chosen to provide information for iontophoretic delivery under physiological conditions. The 0.016 and 0.04 M PBS solutions were used only in the salicylate and BSA experiments for comparison of the present results to those in the literature. The background electrolyte solutions were filtered by Millipore membrane filter before use. Samples were injected into the column hydrodynamically at a pressure of 0.5 psi for 5 s. The applied electrical potential was 10 kV and 6 kV for the 50 and 75 μm I.D. capillary columns, respectively. The inlet of the bare fused silica column was always the anode during capillary electrophoresis except in the preliminary studies. The capillary and sample temperature were maintained at 25 °C. Detection was accomplished using a diode array detector monitoring at 212 and 254 nm. Benzyl alcohol was used as a neutral marker to monitor the electroosmotic flow in the column. Capillary electrophoresis of benzyl alcohol was carried out before and after each experiment and between every other sample run. In a number of experiments, the neutral marker was mixed with the analyte sample so both the marker and analyte were analyzed concurrently to compare the migration times with and without the marker.

Hydrodynamic experiments using pressure separation were performed in the capillary electrophoresis system without the application of an electric field to identify possible interactions between the analyte and the capillary column surface. Experiments were performed by the application of 0.5 psi pressure to drive the analytes and neutral marker across the capillary column under the same experimental conditions as those in the capillary electrophoresis experiments (e.g., same background electrolyte and analyte concentration). 0.5 psi was selected because it resulted in similar migration times for the neutral marker as those obtained in the presence of the electric field.

2.3. Dynamic light scattering

The molecular sizes and diffusion coefficients of BSA, PSS, bevacizumab, and ranibizumab were determined by dynamic light scattering using Malvern Zetasizer® Nano ZS (Malvern Instruments Ltd., United Kingdom). Gold nanoparticles (RM 8011, NIST, MD, USA) were the standard used to qualify the instrument. BSA, PSS, bevacizumab, and ranibizumab solutions at concentrations from 0.05 to 0.3% (w/w) were prepared in 0.04 and/or 0.16 M PBS. The samples were filtered and pipetted into disposable cuvettes, and the average hydrodynamic radii and diffusion coefficients were measured.

2.4. Intrinsic electrophoretic mobility and effective charge calculation

The intrinsic electrophoretic mobilities (μ_i) of the analytes were calculated using the migration times observed for each analyte and those of the electroosmotic flow marker as described previously [19,21] under the assumption of no significant interaction between the analytes and the wall of the capillary column that affects the analyte migration times. The electrophoretic mobility of an analyte is related to its diffusion coefficient according to the Einstein relation for the ideal case (e.g., at infinite dilution):

$$\mu_i = \frac{D_i z_i e}{kT} \quad (1)$$

where k is the Boltzmann constant, e is the elementary charge constant, T is the temperature, z_i is the charge number, and D_i is the diffusion coefficient of the analyte. Eq. (1) does not account for the effects of the migrating ions surrounding the analyte upon its electrophoretic mobility (e.g., relaxation and electrophoretic effects). Due to these effects, the effective charge calculated using Eq. (1) at the ionic strength under physiological conditions could be up