



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.

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### 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  $\mu\text{m}$  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  $\mu\text{m}$  I.D. (BIOTAQ Inc., Washington DC) and 75  $\mu\text{m}$  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  $\mu\text{m}$  I.D. columns, respectively. PBS of 0.016, 0.04, and 0.16 M ionic strength was the background elec-

trolyte 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  $\mu\text{m}$  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 ( $\mu_i$ ) 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

**Table 1**  
Intrinsic electrophoretic mobilities of the analytes.

Analyte	Background electrolyte condition	Intrinsic electrophoretic mobility ( $\times 10^{-4}$ cm <sup>2</sup> /s/V) <sup>a</sup>
Salicylate	0.04 M, PBS	$-2.9 \pm 0.2$
Salicylate	0.016 M, PBS	$-2.8 \pm 0.3$
Lidocaine	0.16 M, PBS	$0.9 \pm 0.1$
Bovine serum albumin	0.16 M, PBS	$-1.7 \pm 0.2$
Bovine serum albumin	0.04 M, PBS	$-1.9 \pm 0.2$
Polystyrene sulfonate	0.04 M, PBS	$-4.4 \pm 0.6$
Bevacizumab	0.16 M, PBS	$-0.9 \pm 0.2$
Ranibizumab	0.16 M, PBS	$-0.28 \pm 0.15$

<sup>a</sup> mean  $\pm$  SD ( $n \geq 4$ ).

to ~20% lower than the ionic charge for a small monovalent ion. Thus, the effective charge of the analyte calculated using Eq. (1) is the effective charge of the Nernst–Einstein relationship under physiological conditions and the ideal case assumption. To take into the account of the interactions between a macromolecule analyte and the surrounding ions, according to the Henry equation, the electrophoretic mobility of the macromolecule is related to its Stokes–Einstein radius and the solution ionic strength:

$$\mu_i = C \frac{\varepsilon \xi_i}{\eta} = C \frac{z_i e}{4\pi\eta R_{SE,i}(1 + \kappa R_{SE,i})} \quad (2)$$

where  $\varepsilon$  is the permittivity and  $\eta$  is the viscosity of the solution medium,  $\kappa$  is the reciprocal of the Debye length, and  $R_{SE,i}$  and  $\xi_i$  are the effective Stokes–Einstein radius and zeta potential of the analyte, respectively.  $C$  is a function of  $\kappa R_{SE,i}$  and varies between 0.67 and 1.0 [25].

### 3. Results and discussion

#### 3.1. Electrophoretic mobility and diffusion coefficient measurements

Table 1 summarizes the intrinsic electrophoretic mobilities of salicylate, lidocaine, BSA, PSS, bevacizumab, and ranibizumab calculated by the migration time data in the capillary electrophoresis experiments. The electrophoretic mobility of salicylate (an anion control) determined using the method in the present study is consistent with the value in the literature ( $-3.6 \times 10^{-4}$  cm<sup>2</sup>/s/V at infinite dilution) [26] and the electrophoretic mobility of lidocaine (a cation control) is lower than that in a previous study ( $1.4 \times 10^{-4}$  cm<sup>2</sup>/s/V in HEPES buffer at pH 7) [23]. The electrophoretic mobility of BSA (a macromolecule control) was also similar to the literature value ( $-2.3 \times 10^{-4}$  cm<sup>2</sup>/s/V in 0.01 M NaCl) [16]. The electrophoretic mobility of PSS in PBS is lower than that expected from the polymer structure. This observation is consistent with previous studies with polyelectrolytes [27–29].

To examine the effect of analyte to capillary column surface interactions upon analyte migration in capillary electrophoresis and test the assumption that this effect does not affect analyte migration times during capillary electrophoresis, hydrodynamic experiments using pressure separation were performed. No significant difference was observed between the migration times of the neutral marker and the macromolecules under pressure driven migration in the columns (e.g., migration times of benzyl alcohol, ranibizumab, and bevacizumab were  $4.27 \pm 0.02$ ,  $4.29 \pm 0.01$ , and  $4.27 \pm 0.01$  min, respectively, mean  $\pm$  SD,  $n \geq 4$ ). These results suggest no significant interactions between the macromolecules and capillary wall surface that would have affected the electrophoretic mobility measurements in the present study.

Table 2 presents the hydrodynamic radii (Stokes–Einstein radii) of BSA, PSS, bevacizumab, and ranibizumab determined using dynamic light scattering. These results are approximately 1.5–2

times larger than the molecular radii estimated using the molecular weight under the assumption of spherical molecular geometry; this is consistent with the general observation of the hydrodynamic radii being larger than the mass equivalent spherical radii of molecules. The diffusion coefficients of BSA, PSS, bevacizumab, and ranibizumab were also determined by dynamic light scattering (Table 2). These diffusion coefficient results are similar to those of BSA, IgG, and Fab, respectively, in previous studies [30–35]. There was no significant difference between the hydrodynamic radius and diffusion coefficient results of BSA in 0.04 and 0.16 M PBS (data not shown).

#### 3.2. Effective charges of the macromolecules

The net effective charges of the analytes were calculated using the electrophoretic mobility data, diffusion coefficients, Stokes–Einstein radii, Eqs. (1) and (2), and are shown in Table 2. The effective charges determined using the Henry equation (Eq. (2)) are generally higher than those calculated under the ideal case assumption (Eq. (1)) because the Einstein relation assumes no influence of the surrounding ions on the electrophoretic mobilities of the macromolecules. The results of salicylate and lidocaine in the control experiments are consistent with their molecular structures; the effective charge of lidocaine was significantly lower than unity in part due to the degree of ionization of lidocaine at pH 7.4. The effective charge of BSA calculated using Eq. (1) is similar to the value reported previously at 0.3 mM and pH 6.8 [35] and the effective charge calculated using Eq. (2) is 1.7 times larger than that in 10 mM NaCl at pH 7.4 [16].

In Table 2, the effective charge of PSS calculated using the Henry equation (Eq. (2)) was lower than that expected from the molecular charge estimated from its structure ( $z \approx -300$  according to the number of monomers in the polymer and assuming 90% sulfonation; manufacturer information). This can be due to factors such as the relaxation effect, counterion condensation, and PSS being a free draining coil rather than a rigid sphere. Without the correction of the electrophoretic effect by the Henry equation, the effective charge of PSS determined using the Einstein relation is an order of magnitude lower than that anticipated from the polymer structure.

Both bevacizumab and ranibizumab are net negatively charged in PBS of pH 7.4 and 0.16 M ionic strength (Table 2), conditions likely encountered in ocular drug delivery. It should be emphasized that the results presented in the table for bevacizumab and ranibizumab are the effective charges of the macromolecules and different from the theoretical net charges of bevacizumab and ranibizumab, which are +6.9 and +2.2 at pH 7.4 (both  $pI \sim 8.8$ ), respectively (product information from Genentech, Inc.). The observed differences between the effective charges and molecular charges calculated from the theoretical isoelectric point ( $pI$ ) and those from the protein sequences can be due to binding of buffer ions to the proteins [16,36] and the differences between the effective and theoretical  $pI$  of proteins under different conditions [37].

#### 3.3. Capillary electrophoresis study for iontophoretic drug delivery

PSS was examined in a previous transscleral iontophoresis study for ocular delivery [12]. PSS is a free draining coil with high negative charge and a structure similar to those of biological polyelectrolytes such as polynucleotides and RNA. Thus, it can be a reasonable model probe for the iontophoretic transport behavior of polynucleotides and RNA. The results in the present study suggest that although PSS (similar to polynucleotides) has high negative charge, the electrophoretic mobility is significantly lower than that expected from the polymer structure and Einstein relation. Thus, to predict the effectiveness of iontophoretic delivery of these

**Table 2**  
Molecular weight (MW), molecular radii, diffusion coefficients, and effective charges of the analytes.

Analyte	MW	Hydrodynamic radius (nm) <sup>a</sup>	Diffusion coefficient ( $\times 10^{-6}$ cm <sup>2</sup> /s)	Effective charge <sup>b</sup>	Effective charge <sup>c</sup>
Salicylate <sup>d</sup>	137 (anion)	N.D. <sup>e</sup>	8.8 <sup>f</sup>	−0.8	N.D. <sup>e</sup>
Lidocaine <sup>d</sup>	235 (cation)	N.D. <sup>e</sup>	4.6 <sup>g</sup>	+0.5	N.D. <sup>e</sup>
Bovine serum albumin	$6.6 \times 10^4$	4.8	0.56 <sup>h</sup>	−9	−36
Polystyrene sulfonate	$6.7 \times 10^4$	6.9	0.39 <sup>h</sup>	−29	−150
Bevacizumab	$1.5 \times 10^5$	6.5	0.41 <sup>h</sup>	−5	−45
Ranibizumab	$4.8 \times 10^4$	4.1	0.67 <sup>h</sup>	−1.1	−7

<sup>a</sup> From dynamic light scattering measurements; average values from at least three different solutions, each with three measurements. For comparison, the hypothetical molecular radii estimated from the relationship:  $R_{SE,i} = (3M_w / (4\pi N_A \rho))^{1/3}$  which assumes the molecules are hard spheres are 3.0, 3.0, 3.9, and 2.7 nm for BSA, PSS, bevacizumab, and ranibizumab, respectively.

<sup>b</sup> Estimated using Eq. (1). For BSA and salicylate, 0.04 M PBS electrophoretic mobility data were used.

<sup>c</sup> Estimated using Eq. (2). For BSA, 0.04 M PBS electrophoretic mobility data were used.

<sup>d</sup> Salicylate pK<sub>a</sub> = 3.0; lidocaine pK<sub>a</sub> = 7.9.

<sup>e</sup> Not determined.

<sup>f</sup> From [40] and corrected for water viscosity and temperature changes at 25 and 37 °C.

<sup>g</sup> Unpublished experimental diffusion coefficient determined using the method in [40].

<sup>h</sup> From dynamic light scattering measurements at 25 °C; average values from at least three different solutions, each with three measurements.

polyelectrolytes, the influence of the surrounding ions on their electrophoretic mobilities such as electrophoretic effect should be considered.

Bevacizumab and ranibizumab are anti-VEGF agents that have been used in the treatment of eye diseases. Due to the promise these agents offer as posterior eye disease therapies, the delivery of these important drugs to the back of the eye has been a recent research interest. Ocular iontophoresis was proposed for the delivery of these therapeutic agents in eye disease treatments [33,38] but the electrophoretic mobilities and effective charges of these agents are not well defined. The results in the present study indicate that bevacizumab and ranibizumab have low effective charge densities under the physiological condition represented in this study (i.e., pH 7.4 and 0.16 M ionic strength). The low charge densities of bevacizumab and ranibizumab and the corresponding electrophoretic mobilities suggest that these macromolecules would not be significantly enhanced by the mechanism of electrophoresis (direct electric field effect) during iontophoresis in drug delivery. As a result, electroosmosis is likely to be the dominant flux enhancing mechanism in the iontophoretic delivery of these macromolecules. Iontophoretic drug delivery of the anti-VEGF antibody and Fab could be most effective from the anode to cathode (i.e., anodal iontophoresis), e.g., in transscleral iontophoresis. These results are consistent with those observed in a previous transscleral iontophoretic study of bevacizumab [12]. The results in the present study can also provide useful information in the design of other drug delivery systems for bevacizumab and ranibizumab in the treatment of eye diseases [39].

#### 4. Conclusion

The electrophoretic mobilities of salicylate, lidocaine, and BSA determined using capillary zone electrophoresis were generally consistent with the values reported in the literature. Both bevacizumab and ranibizumab are net negatively charged and have low charge densities under the physiological condition. Thus, electroosmosis is likely to be a significant factor in iontophoretic delivery of bevacizumab and ranibizumab. PSS has high negative charge and the main iontophoretic delivery mechanism is likely to be electrophoresis (direct electric field effect). The utility of capillary electrophoresis to characterize drug electrical properties in the development of iontophoretic drug delivery was demonstrated.

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