16th Week

Electroosmosis

The inside wall of a fused-silica capillary is covered with silanol (Si OH) groups, which have a negative charge ($Si—O^-$) above pH 3. Figure 25-24a shows the *electric double layer* (Box 16-3) at the capillary surface. The double layer consists of fixed negative charges on the wall and excess cations near the wall. A tightly adsorbed, immobile layer of cations partially neutralizes the negative charge. The remaining negative charge is neutralized by mobile cations in the *diffuse part of the double layer* in solution near the wall. The thickness of the diffuse part of the double layer ranges from ~10 nm when the ionic strength is 1 mM to ~0.3 nm when the ionic strength is 1 M.

In an electric field, cations are attracted to the cathode and anions are attracted to the anode (Figure 25-24b). Excess cations in the diffuse part of the double layer impart net momentum toward the cathode. This pumping action, called **electroosmosis** (or *electroendosmosis*), is driven by cations within ~10 nm of the walls and creates uniform pluglike *electroosmotic flow* of the entire solution toward the cathode (Figure 25-25a). This process is in sharp contrast with *hydrodynamic flow*, which is driven by a pressure difference. In hydrodynamic flow, the velocity profile through a cross section of the fluid is parabolic: It is fastest at the center and slows to 0 at the walls (Figure 25-25b and Color Plate 30).

Ions in the diffuse part of the double laye adjacent to the capillary wall are the "pur that drives electroosmotic flow.

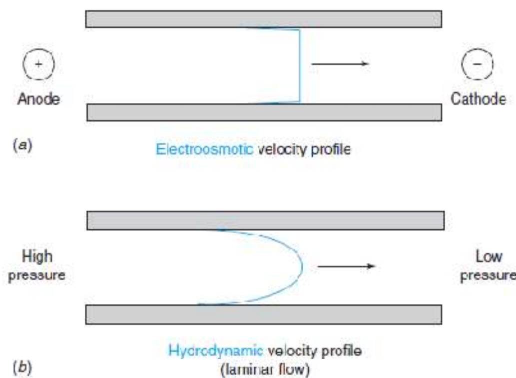


FIGURE 25-25 (*a*) Electroosmosis give uniform flow over more than 99.9% of the cross section of the capillary. The speed decreases immediately adjacent to the cap wall. (*b*) Parabolic velocity profile of hydrodynamic flow (also called *laminar flo* with the highest velocity at the center of th tube and zero velocity at the walls. Experimentally observed velocity profiles a shown in Color Plate 30.

Electroosmotic velocity is measured by adding to the sample a *neutral* molecule to which the detector responds.

$$\text{Electroosmotic velocity} = \frac{\text{distance from injector to detector}}{\text{migration time of neutral molecule}}$$

The constant of proportionality between electroosmotic velocity, u_{eo}, and applied field is called *electroosmotic mobility*, μ_{eo}.

Electroosmotic mobility:

$$u_{eo} = \mu_{eo}E \qquad \textbf{(25-10)}$$

↑ Electroosmotic mobility (units = $m^2/[V \cdot s]$)

Electroosmotic mobility is proportional to the surface charge density on the silica and inversely proportional to the square root of ionic strength. Electroosmosis decreases at low pH ($Si—O^- \rightarrow Si—OH$ decreases surface charge density) and high ionic strength. At pH 9 in 20 mM borate buffer, electroosmotic flow is ~2 mm/s. At pH 3, flow is reduced by an order of magnitude.

Uniform electroosmotic flow contributes to the high resolution of capillary electrophoresis. Any effect that decreases uniformity creates band broadening and decreases resolution. The flow of ions in the capillary generates heat (called *Joule heating*) at a rate of I^2R joules per second, where I is current (A) and R is the resistance of the solution (ohms) (Section 13-1). Most of the capillary in Figure 25-22 is in a water-thermostated compartment necessary for temperature control inside the capillary.[29] Typically, the centerline of the capillary channel is 0.02 to 0.3 K hotter than the edge of the channel. Lower viscosity in the warmer region disturbs the flat electroosmotic profile of the fluid. Joule heating is not a serious problem in a capillary tube with a diameter of 50 μm, but the temperature gradient would be prohibitive if the diameter were ≥1 mm. Some instruments cool the capillary to reduce the electrical conductivity of solution inside the capillary and prevent runaway Joule heating.

The capillary must be thin enough to dissipate heat rapidly. Temperature gradients disturb the flow and reduce resolution.

Mobility

The *apparent* (or observed) *mobility*, μ_{app}, of an ion is the sum of the electrophoretic mobility of the ion plus the electroosmotic mobility of the solution.

Apparent mobility:

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad \textbf{(25-11)}$$

For an analyte *cation* moving in the same direction as the electroosmotic flow, μ_{ep} and μ_{eo} have the same sign, so μ_{app} is greater than μ_{ep}. Electrophoresis transports *anions* in the opposite direction from electroosmosis (Figure 25-24b), so for anions the two terms in Equation 25-11 have opposite signs. At neutral or high pH, brisk electroosmosis transports anions to the *cathode* because electroosmosis is usually faster than electrophoresis. At low pH, electroosmosis is weak and anions may never reach the detector. If you want to separate anions at low pH, you can reverse the polarity to make the sample end negative and the detector end positive.

The apparent mobility, μ_{app}, of a particular species is the net speed, u_{net}, of the species divided by the electric field, E:

$$\text{Speed} = \frac{\text{distance to detector}}{\text{migration time}} = \frac{L_d}{t}$$

$$\text{Electric field} = \frac{\text{applied voltage}}{\text{capillary length}} = \frac{V}{L_t}$$

Apparent mobility:

$$\mu_{app} = \frac{u_{net}}{E} = \frac{L_d/t}{V/L_t} \qquad \textbf{(25-12)}$$

where L_d is the length of column from injection to the detector, L_t is the total length of the column from end to end, V is the voltage applied between the two ends, and t is the time required for solute to migrate from the injection end to the detector. Electroosmotic flow is measured by adding an ultraviolet-absorbing neutral solute to the sample and measuring its *migration time*, $t_{neutral}$, to the detector.

For quantitative analysis, use

$$\frac{\text{Peak area}}{\text{Migration time}}$$

For quantitative analysis by electrophoresis, *normalized peak areas* are required. The normalized peak area is the measured peak area divided by the migration time. In chromatography, each analyte passes through the detector at the same rate, so peak area is proportional to the quantity of analyte. In electrophoresis, analytes with different apparent mobilities pass through the detector at different rates. The higher the apparent mobility, the shorter the migration time and the less time the analyte spends in the detector. To correct for time spent in the detector, divide the peak area for each analyte by its migration time.

Electroosmotic mobility is the speed of the neutral species, $u_{neutral}$, divided by the electric field:

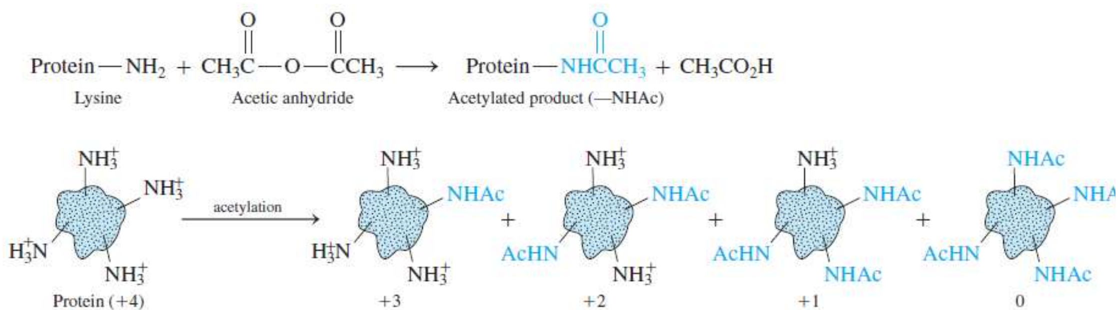
Electroosmotic mobility:

$$\mu_{eo} = \frac{u_{neutral}}{E} = \frac{L_d/t_{neutral}}{V/L_t} \qquad \textbf{(25-13)}$$

The *electrophoretic mobility* of an analyte is the difference $\mu_{app} - \mu_{eo}$.

For maximum precision, mobilities are measured relative to an internal standard. Absolute variation from run to run should not affect relative mobilities, unless there are time-dependent (nonequilibrium) interactions of the solute with the wall.

For molecules of similar size, the magnitude of the electrophoretic mobility increases with charge. A protein "charge ladder" is a synthetic mixture made from a single protein with many different charges.[30] For example, we can obtain such a mixture by acetylating variable numbers of lysine amino acid side chains (Table 9-1) to reduce their charge from +1 ($R—NH_3^+$) to 0 ($R—NHC(=O)CH_3$).



Lysine $—NH_2$ groups have $pK_a \approx 10.3$. At pH 8.3, 99% of these groups are protonated ($—NH_3^+$). Acetylation gives a mixture with every possible number of modified amino groups from 0 to the total number of lysine residues. This mixture gives the electropherogram in Figure 25-26 with a series of nearly evenly spaced peaks. Each molecule has approximately the same size and shape (and therefore nearly the same friction coefficient) but a different charge.

Problem 25-41 shows how to find
of unmodified protein from the ch

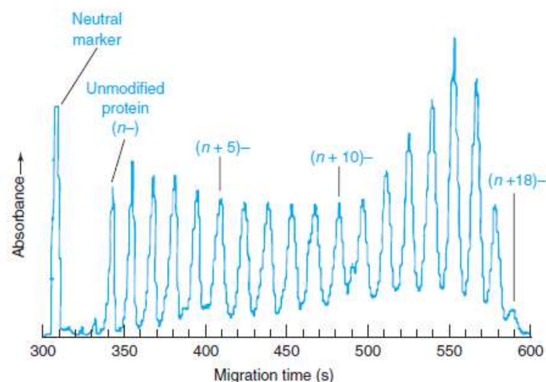


FIGURE 25-26 Protein charge la carbonic anhydrase was acetylated t species with charges of $n-$ (unacety $(n + 1)-$, $(n + 2)-$, . . . , $(n + 18)-$ acetylated). Electrophoresis was carri pH 8.3 at 2.50×10^4 V in a capillary length of 0.840 m and a distance to of 0.640 m. The neutral, ultraviolet-a marker used to measure electroosm was mesityl oxide, $(CH_3)_2C=CHC(=$ [From M. K. Menon and A. L. Zydney, "De Effective Protein Charge by Capillary Electr *Anal. Chem.* **2000**, *72*, 5714.]

Number of plates: $N = \frac{L_d^2}{\sigma^2}$

L_d = distance to detector
σ = standard deviation of Gaussian band
L_t = total length of column

Theoretical Plates and Resolution

Consider a capillary of length L_d from the inlet to the detector. In Section 22-4, we defined the number of theoretical plates as $N = L_d^2/\sigma^2$, where σ is the standard deviation of the band. If the only mechanism of zone broadening is longitudinal diffusion, the standard deviation was given by Equation 22-26: $\sigma = \sqrt{2Dt}$, where D is the diffusion coefficient and t is the migration time ($= L_d/u_{net} = L_d/[\mu_{app}E]$). Combining these equations with the definition of electric field ($E = V/L_t$, where V is the applied voltage) gives an expression for the number of plates:

Number of plates: $$N = \frac{\mu_{app}V}{2D}\frac{L_d}{L_t} \qquad (25\text{-}14)$$

How many theoretical plates might we hope to attain? Using a typical value of $\mu_{app} = 2 \times 10^{-8}\ m^2/(V \cdot s)$ (derived for a 10-min migration time in a capillary with $L_t = 60$ cm, $L_d = 50$ cm, and 25 kV) and using diffusion coefficients from Table 22-1, we find

$$\text{For } K^+\text{:}\quad N = \frac{[2 \times 10^{-8}\ m^2/(V \cdot s)][25\,000\ V]}{2(2 \times 10^{-9}\ m^2/s)}\frac{0.50\ m}{0.60\ m} = 1.0 \times 10^5 \text{ plates}$$

$$\text{For serum albumin:}\quad N = \frac{[2 \times 10^{-8}\ m^2/(V \cdot s)][25\,000\ V]}{2(0.059 \times 10^{-9}\ m^2/s)}\frac{0.50\ m}{0.60\ m} = 3.5 \times 10^6 \text{ plates}$$

For the small, rapidly diffusing K^+ ion, we expect 100 000 plates. For the slowly diffusing protein serum albumin (FM 66 000), we expect more than 3 million plates. High plate count means that bands are very narrow and resolution between adjacent bands is excellent.

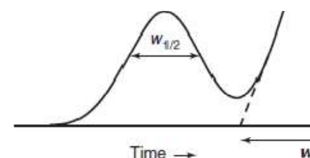
Under special conditions in which a reverse hydrodynamic flow was imposed to slow the passage of analytes through the capillary, up to 17 million plates were observed in the separation of small molecules![31]

In reality, additional sources of zone broadening include the finite width of the injected band (Equation 22-32), a parabolic flow profile from heating inside the capillary, adsorption of solute on the capillary wall (which acts as a stationary phase), the finite length of the detection zone, and mobility mismatch of solute and buffer ions that leads to nonideal electrophoretic behavior. If these factors are properly controlled, $\sim 10^5$ plates are routinely achieved.

their width.

25-7 Conducting Capillary Electrophoresis

Clever variations of electrophoresis allow us to separate neutral molecules as well as ions, to separate optical isomers, and to lower detection limits by up to 10^6.



$$\text{Resolution} = \frac{\Delta t}{w_{av}} = \frac{0.589\Delta t}{w_{1/2av}}$$

$$\text{Resolution} = \frac{\sqrt{N}}{4}(\gamma - 1)$$

N = plate number

γ = separation factor

$$= \frac{\text{speed of faster species}}{\text{speed of slower species}}$$

Controlling the Environment Inside the Capillary

The inside capillary wall controls electroosmotic velocity and provides undesired adsorption sites for multiply charged molecules, such as proteins. A fused-silica capillary should be prepared for its *first* use by washing for 1 h with 1 M NaOH at a flow rate of ~4 column volumes/min, followed by 1 h with water, followed by 1 h with 6 M HCl, followed by 1 h with run buffer.[32] NaOH is thought to generate Si—OH groups on the silica surface and HCl removes metal ions from the surface. For subsequent use at high pH, wash for ~10 s with 0.1 M NaOH, followed by deionized water, and then by at least 5 min with run buffer.[33] If the capillary is being run with pH 2.5 phosphate buffer, wash between runs with 1 M phosphoric acid, deionized water, and run buffer.[34] When changing buffers, allow at least 5 min of flow for equilibration. For the pH range 4–6, at which equilibration of the wall with buffer is slow, the capillary needs frequent regeneration with 0.1 M NaOH if migration times become erratic. Buffer in both reservoirs should be replaced periodically because ions become depleted and because electrolysis raises the pH at the cathode and lowers the pH at the anode. The capillary inlet should be ~2 mm away from and below the electrode to minimize entry of electrolytically generated acid or base into the column.[35] Stored capillaries should be filled with distilled water.

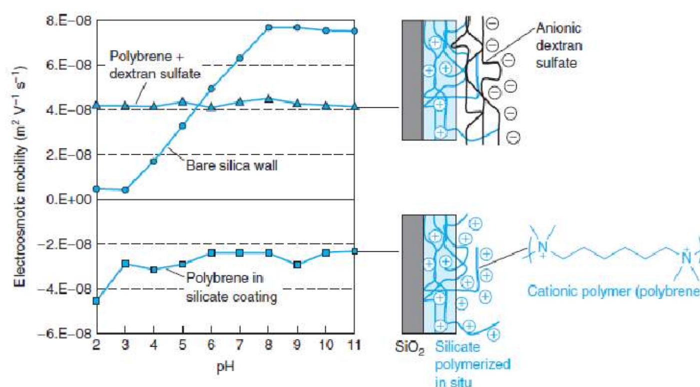
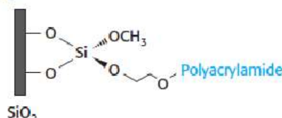


FIGURE 25-28 Effect of wall coating on electroosmotic mobility. Bare silica has little charge below pH 3 and high negative charge above pH 8. Polybrene cation embedded in silicate (lower structure) gives nearly constant positive charge to the wall. Anionic dextran sulfate adsorbed on polybrene (upper structure) gives constant negative charge to the wall. The bare silica wall goes from having little charge at pH 3 to full negative charge at pH 8. The wall behaves as if it has two populations of Si—OH groups with a total surface density of ~4.3×10^{-7} mol/m^2. One-fourth of the silanols have $pK_a = 4.0$ and three-fourths have $pK_a = 5.5$.[36] [From M. R. N. Monton, M. Tomita, T. Soga, and Y. Ishihama, "Polymer Entrapment in Polymerized Silicate for Preparing Highly Stable Capillary Coatings for CE and CE–MS," *Anal. Chem.* **2007**, *79*, 7838.]

Covalent coating helps prevent protein from sticking to capillary and provides reproducible migration times:



Different separations require more or less electroosmotic flow. Small anions with high mobility and highly negatively charged proteins require brisk electroosmotic flow or they will not travel toward the cathode. At pH 3, there is little charge on the silanol groups and little electroosmotic flow. At pH 8, the wall is highly charged and electroosmotic flow is strong. The graph in Figure 25-28 shows that electroosmotic mobility in a bare silica capillary is small and positive below pH 3. Mobility increases and reaches a high, steady value above pH 8.

Proteins with many positively charged substituents can bind tightly to negatively charged silica. To control this, 30–60 mM diaminopropane (which gives $^+H_3NCH_2CH_2CH_2NH_3^+$) may be added to the run buffer to neutralize charge on the wall. The wall charge can be reduced to near 0 by covalent attachment of silanes with neutral, hydrophilic substituents. However, many coatings are unstable under alkaline conditions.

You can reverse the direction of electroosmotic flow by adding a cationic surfactant, such as didodecyldimethylammonium bromide, to the run buffer.[37] This molecule has a positive charge at one end and two long hydrocarbon tails. The surfactant coats the negatively charged silica, with the tails pointing away from the surface (Figure 25-29). A second layer of surfactant orients itself in the opposite direction so that the tails form a nonpolar hydrocarbon layer. This *bilayer* adheres tightly to the wall of the capillary and effectively reverses the wall charge from negative to positive. Buffer anions create electroosmotic flow from cathode to anode when voltage is applied. Electroosmotic flow is in the direction opposite that shown in Figure 25-24. Best results are obtained when the capillary is freshly regenerated for each run.

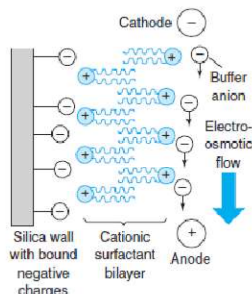


FIGURE 25-29 Charge reversal created by a cationic surfactant bilayer coated on the capillary wall. The diffuse part of the double layer contains excess anions, and electroosmotic flow is opposite that shown in Figure 25-24. The

Figure 25-28 shows a more stable cationic coating formed by embedding the cationic polymer polybrene in a silicate layer formed in situ on the capillary wall. The graph shows that electroosmotic flow is nearly constant in the pH range 2–11 and opposite that of bare silica. A stable, pH-independent negative surface can be made by adsorption of the anionic polymer dextran sulfate on the cationic polybrene surface.