Capillary electrophoresis (CE) as an analytical technique is now two decades old, and commercial CE instruments have been available for more than 10 years. Initial expectations for CE were high, and scientists anticipated that CE would rival high performance liquid chromatography (HPLC) as a general separation technique. This prediction has not come to pass, due in part to CE’s inability to deliver the desired performance for many routine applications when compared with liquid chromatography. However, CE is finding success in areas in which existing technologies fall short. A high-profile example is the replacement of gel-based deoxyribonucleic acid (DNA) sequencers by automated multicapillary-based instruments, which yielded the first draft of the human genome sequence last year. On a quieter front, CE increasingly is used as a replacement for gel-based analyses of biopharmaceuticals. Automation and quantitative analysis are key advantages that persuade scientists in the biopharmaceutical industry to trade their polyacrylamide gels for CE systems.

The California Separation Science Society sponsored a one-day symposium on CE in biotechnology, and it focused on the practical concerns of CE analysis of protein products. The symposium addressed CE applications in various aspects of drug development, including discovery, process development, and quality control. The symposium was held in San Francisco, California, on 18 August 1999. A second symposium was held in San Diego, California, on 28–29 August 2000. Organized by Tony Chen and Wassim Nashabeh of Genentech (South San Francisco, California), SungAe Park of Amgen ( Thousand Oaks, California), and Claudia Jochheim of Immunex (Seattle, Washington), the San Diego meeting was expanded to include CE of small molecule pharmaceuticals as well as biopharmaceuticals. It also presented CE applications in various aspects of drug development, including discovery, process development, and quality control. This month’s installment of “Directions in Discovery” will review new developments highlighted at the San Diego meeting and the practical issues researchers face when applying CE to analysis of protein products.

Analyzing Protein Biopharmaceuticals: Replacing Slab Gel Techniques
The major focus of both the 1999 and the 2000 symposia was the use of CE as an automated approach to replacing classical slab gel techniques for analyzing recombinant protein products. The availability of commercial kits and reagents for both of these techniques helps simplify method development.

The capillary alternative to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) uses entangled-polymer sieving solutions to achieve size-based separations of proteins. The hydrophilic polymers used in these solutions form dynamic networks that mimic the sieving action of gels but are replaced after each analysis by purging the capillary and replenishing it with fresh polymer solution. The polymers also are transparent in the low UV region, enabling high-sensitivity on-line detection of the SDS-protein complexes without staining. Using this technique for purity determination of monoclonal antibodies was discussed in the February installment of “Directions in Discovery” (1).

Stacey Ma of Genentech discussed the application of entangled-polymer sieving CE to the analysis of size variants in recombinant monoclonal antibody preparations. Detection by UV absorbance at
220 nm provided sensitivity equivalent to Coomassie Blue staining, and it could be used to detect major size variants such as light-heavy chain fragments. This approach was also useful for monitoring yields of recombinant human Fab antibody fragments by the derivation of proteins with polyethylene glycol and for determining monoclonal antibody glycosylation occupancy.

Jochheim (2) also described the resolution of glycoforms by entangled-polymer sieving. Glycosylated proteins exhibit slower migration in polymer networks, probably because of alterations in protein size and shape due to the presence of carbohydrate moieties and to reduced SD S binding. Jochheim was able to resolve glycoforms of granulocytoe macrophage colony stimulating factor from nonglycosylated protein and to separate two groups of O- and N-linked glycoforms (Figure 1).

Although UV detection is suitable for quantitating major components in protein preparations, it lacks sufficient sensitivity for minor species. In SD S–PAGE, silver staining is required for these applications, because sensitivity is increased 10- to 100-fold compared with Coomassie staining. To solve this problem, Ma and her associates used derivatization to introduce a fluorophore that can be detected with high sensitivity using laser-induced fluorescence detection. Using a simple derivatization procedure, the sample was reacted with 5-carboxytetramethylrhodamine before complexation with SD S, and fluorescent products were detected using excitation at 488 nm with an argon ion laser. Surprisingly, minimal peak broadening was observed after derivatization, possibly because of the accessibility of the lysine and arginine side chains in these monoclonal antibody molecules. Fluorescence detection of the derivatives provided a 100-fold increase in sensitivity relative to UV detection, and this increase enabled quantitation of minor impurities in monoclonal antibody preparations with sensitivity equivalent to or better than silver staining. An important task in the validation of this method was demonstrating that the distribution of protein variants was similar to that of unlabeled material.

Louie and co-workers (3) from Genetech described using this methodology as a stability-indicating assay for monoclonal antibody, Fab antibody fragments, and a recombinant E. coli-derived polypeptide (Figure 2). This group demonstrated that relative increases in aggregation monitored by CE–SD S with laser-induced fluorescence detection were consistent with those found when analyzing the same samples by high performance size-exclusion chromatography run with a mobile phase that contained SD S. Guo and colleagues from BASF Bioresearch Corp. (Worcester, Massachusetts) discussed validation of an entangled-polymer sieving method for analyzing recombinant monoclonal antibody product-related variants and impurities. The Genentech group evaluated detection of native fluorescence using a frequency-doubled krypton laser to excite tryptophan residues at 284 nm. They used this approach to monitor Chinese hamster ovary host cell protein impurities and to obtain an intermediate detection sensitivity between UV and laser-induced fluorescence of tetramethylrhodamine-labeled proteins.

Isolelectric focusing, which separates proteins on the basis of their isoelectric points in a stable pH gradient, has been the method of choice for identifying charge variants such as deamidation products, clips, C-terminal process variants, and glycoforms that vary in sialic acid content. Researchers have described several approaches to performing capillary isoelectric focusing, most of which rely upon a means to transport focused protein zones past a fixed detection point at the distal end of the capillary. The transport force can be electrophoretic (chemical mobilization), hydraulic (pressure, vacuum, or gravity mobilization), or electroendosmotic (electroosmotic flow mobilization). At last year’s meeting in San Francisco, Jochheim (4) discussed the successful use of capillary isoelectric focusing with electrophoretic mobilization for recombinant human tumor necrosis factor.

The mobilization step can limit resolution in capillary isoelectric focusing and contribute to poor results for proteins that tend to aggregate and precipitate under isoelectric conditions. Janusz Pawliszyn of the University of Waterloo (Waterloo, Ontario, Canada) developed an alternative approach, which has been commercialized by Convergent Bioscience Ltd. (Toronto, Ontario, Canada). In this technique, called imaged capillary isoelectric focusing, proteins and carrier ampholytes are focused in a UV-transparent capillary, and the entire capillary is imaged onto a charge-coupled device camera. No mobilization step is required, and the focusing process can be monitored in real time as an aid in method development.

Joseph Ratto and co-workers (5) from Amgen described using imaged capillary isoelectric focusing to monitor monoclonal antibodies.

### Automation and Quantitative Analysis

Automation and quantitative analysis are key advantages that persuade scientists in the biopharmaceutical industry to trade their polyacrylamide gels for CE systems. This provides a 100-fold increase in sensitivity relative to UV detection, and this is increased when compared with silver staining. An important task in the validation of this method was demonstrating that the distribution of protein variants was similar to that of unlabeled material. This approach was also useful for monitoring yields of recombinant human Fab antibody fragments by the derivation of proteins with polyethylene glycol and for determining monoclonal antibody glycosylation occupancy.

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### Figure 1: Capillary electrophoresis-SDS nongel sieving separation of granulocyte macrophage colony stimulating factor glycoforms.

(Figure provided by Claudia Jochheim of Immunex Corp. [2])

### Figure 2: Entangled-polymer sieving of SDS complexes of a nonreduced E. coli-derived polypeptide after 12 and 18 months storage at 5 °C. Stability samples are compared with reference material stored at −70 °C. (Figure provided by Glenn Hunt and Sean Richardson of Genentech, Inc. [3])
antibody glycoforms and was able to resolve as many as 16 isoforms (Figure 3). The resolution was comparable to gel-based isoelectric focusing, and the authors obtained precision values of 0.22% relative standard deviation (RSD) for pI and 2.86% RSD for area percentage.

Jochheim (2) used capillary isoelectric focusing to resolve one major component and five to eight minor components of yeast-produced granulocyte macrophage colony stimulating factor and found that capillary isoelectric focusing reduced the precipitation and reproducibility problems encountered with conventional isoelectric focusing methods (Figure 4).

Capillary zone electrophoresis (CZE) also has been used to separate protein charge variants. This approach may be preferable for proteins that exhibit precipitation problems with isoelectric focusing. Ma (6) described the use of CZE for monitoring charge heterogeneity of recombinant monoclonal antibodies and as a fast identity test to differentiate various monoclonal antibodies (Figure 5). These analyses were performed using an ε-amino caproic acid-acetic acid buffer (pH 4.5) with a capillary covalently coated with polyacrylaminoethoxyethanol. The buffer was supplemented with 0.1% hydroxypropylmethylcellulose, presumably to eliminate any residual protein-wall adsorption.

Bernice Yeung of Amgen analyzed sialic acid isoforms of acidic glycoproteins using similar conditions (ε-amino caproic acid buffer and Tween [pH 4.4] using a polyvinyl alcohol–coated capillary) and observed isoform patterns similar to those obtained with capillary isoelectric focusing but with improved precision.

Electrophoretic mobility of a protein in free solution is affected by molecular size and shape, so CZE can be used to monitor changes in protein conformation. Steven Berkowitz of Biogen (Cambridge, Massachusetts) used CZE with a borate buffer that contained SDS and a proprietary amine additive to resolve a series of metastable conformational states of an antibody that had been subjected to elevated temperature for varying times. The formation of borate complexes with carbohydrate moieties in the borate-SDS buffer system enhanced the separation of glycoforms of the fully denatured protein.

At the close of the formal presentations, a workshop gave speakers and attendees the opportunity to discuss practical issues in CE analysis of protein biopharmaceuticals. Topics included tips for sample preparation, using additives for suppression of protein adsorption and electroosmotic flow, and using low-conductivity buffers. One participant noted that conversion of a gel-based assay to a CE-based assay can reduce labor costs by 30–50%. An important limitation of CE is its difficulty in characterizing the many peaks resolved by CE techniques because of the small peak volumes and problems in collecting material for analysis. On-line coupling of CE and mass spectrometry (MS) may help resolve this issue, but the poor compatibility of most protein CE buffers with MS still will present obstacles.

Analyzing Small Molecules: Toward Generic Methods

The lack of widespread acceptance of CE as a tool for analysis of small-molecule pharmaceuticals can be traced in part to operator experience, lack of technical knowledge, and a perception that CE methods lack precision and ruggedness. However, it was clear from several presentations in this part of the symposium that laboratories that make the investment in understanding CE technology are able to apply it successfully for routine analysis. In this regard, the development of standard or generic methods reduces development costs and greatly facilitates CE application to a wide range of analytes.

Kevin Altria of Glaxo Wellcome (Ware, United Kingdom) described four standard CE methods his company currently uses.

![Figure 3](image-url)
For basic drugs, he uses a CZE method with a pH 2.5 phosphate buffer. Acidic drugs are analyzed by CZE using 15 mM borate (pH 9.5). Analytes are characterized by their migration relative to two internal standards: imidazole and aminobenzoate. For mixtures of acidic and basic analytes, he prefers micellar electrokinetic chromatography (MEKC) using a 20 mM borate buffer containing 15 mM β-cyclodextrin and 75 mM lithium dodecyl sulfate (the lithium salt of the surfactant generates a lower operating current than the more commonly used sodium salt). Benzoic acid and 4-hydroxyacetophenone are the internal standards used with MEKC. Recently, his laboratory introduced a microemulsion chromatography method that has broader utility than the earlier CZE and MEKC methods. The run buffer for this technique comprises sodium borate, SDS, octanol, and butanol added as a co-surfactant. Both the M EKC and the microemulsion methods are useful for analytes with poor solubility in water. These standard methods have been applied at Glaxo Wellcome for identity tests, batch profiling, formulation assays, and drug substance assays.

A generic method for resolution of drug enantiomers was described by Jeff Chapman of Beckman Coulter (Fullerton, California). The buffer used in this method comprises 25 mM phosphate (pH 2.5) and one of a family of highly sulfated cyclodextrins. Cyclodextrins are cyclic polysaccharides that can form inclusion complexes with analytes, and differential inclusion is the basis for chiral discrimination. The highly sulfated cyclodextrin additives comprise 11, 12, or 13 sugar groups, which have cavities of increasing diameter. These additives allow the highly sulfated cyclodextrin to be tailored to the size of the analyte for efficient complexation and chiral selection. The negative charge of the sulfated cyclodextrins makes them highly soluble and confers anodic mobility to complexed analytes to enable separation of both neutral and ionic compounds in the same analysis. The low-pH phosphate buffer reduces electroosmotic flow and suppresses wall interactions of basic analytes. Chapman used 1,3,6,8-pyrenetetrasulfonate as a reference marker because it does not interact with the highly sulfated cyclodextrin additives. Of 160 chiral drugs investigated, he was able to resolve enantiomers for 92 of 92 basic drugs, 56 of 58 neutral drugs, and 8 of 10 acidic drugs with resolution greater than 2.

Figure 4: Separation of granulocyte macrophage colony stimulating factor charge variants using (a) imaged capillary isoelectric focusing, (b) conventional capillary isoelectric focusing, and (c) slab gel isoelectric focusing. (Figure provided by Claudia Jochheim of Immunex Corp. [2])

Figure 5: CZE analysis of recombinant monoclonal antibodies. Capillary: 47 cm × 50 μm BioCap XL coated capillary (Bio-Rad Laboratories); buffer: 45 mM L-aminocaproic acid–acetic acid (pH 4.5), 0.1% hydroxypropylmethylcellulose; temperature: 20 °C; voltage: 30 kV, normal polarity; injection: hydrodynamic. (Figure provided by Stacey Ma of Genentech, Inc. [6])
The formal presentations about CE analysis of small-molecule pharmaceuticals concluded with a workshop to address practical considerations. This workshop was led by Altria, and his extensive CE experience made for an informative session. Topics discussed included capillary preparation and conditioning, strategies for increasing sensitivity, cautions about the effects of ion depletion and electrode reactions, and tips for achieving good injection precision.

**Electroosmotic Flow:**

Dr. Jekyll and Mr. Hyde personify the two faces of electroendosmotic flow in electromigration separations.

In a presentation by Lilian Clohs of Nortrans Pharmaceuticals (Vancouver, British Columbia, Canada). Nortran is developing drugs for pathologies mediated by cellular ion channels, particularly cardiac arrhythmia. Nortran’s analytical chemistry department has adopted CE as the method of choice for purity and enantiomeric excess determinations of new chemical entities, stability monitoring, pharmacokinetic and bioavailability studies, and protein-binding studies. CE was chosen for its rapid analysis times, good accuracy and precision, and ease of method development and transfer. A few standard methods served for a variety of applications. For purity determinations and pharmacokinetic studies, a simple method using 100 mM sodium phosphate buffer (pH 2.5) and normal polarity at 25 kV sufficed. The method’s sensitivity was satisfactory for Nortran’s applications, and matrix interferences were absent in extracts prepared from a variety of biological fluids. For determining enantiomeric excess, Clohs used a triethylamine phosphate buffer supplemented with highly sulfated cyclodextrin in reversed polarity.

Capillary electrophoretic principles have been used for performing high-throughput separations in miniaturized multichannel systems, and two of these microfabricated devices have been introduced recently. Jacqueline Thorne of R.W. Johnson PRI (Raritan, New Jersey) described the use of one of these, the Aclara Biosciences LabCard (Mountain View, California), for high-throughput screening. This instrument is a 96-channel device with integrated fluidic transfer, separation, and confocal fluorescence detection components. The goal of Thorne’s exploratory technology group is to develop the capacity for performing 100,000 enzyme- and cell-based assays per day. Thorne selected the LabCard for her laboratory’s applications because of its parallel processing capability and short assay times. Enzyme assays included kinase, phosphorylase, and protease. These assays were convenient to perform by electrophoretic separations because protease cleavage and changes in phosphorylation typically alter the mobilities of the substrate. Correlations with conventional in-tube assays were good (correlation coefficients [R] were greater than or equal to 0.98) and assay times were as short as 4 s.

Thorne applied the LabCard system to cell-based assays to determine efficacy, permeability, and toxicity. She described an apoptosis assay in which DNA damage induced by etoposide (a topoisomerase inhibitor) was monitored by changes in the migration pattern of a double-stranded DNA ladder.

**CE in the Development of a Gene Therapy Product**

Adenoviruses are DNA-containing viruses that have been widely used as vectors for gene therapy, and they account for 20% of gene therapy clinical trials. They are useful vectors because they are easy to produce in high titer, are highly infectious, have a broad host range, and cannot integrate into the host genome. The last characteristic makes adenoviruses useful for short-term therapy.

Joseph Traina of Berlex Biosciences (Richmond, California) described using CE to characterize an adenovirus vector carrying the gene for fibroblast growth factor 4. The fibroblast growth factor gene product enhances angiogenic processes, and the vector is intended for genetic therapy of ischemia. Traina used CE to characterize the intact virus and viral components. He analyzed the virus particle by CZE using a 25 mM phosphate buffer (pH 7) with a polyvinyl alcohol-coated capillary. In addition to the main viral peak, he observed multiple isoforms that were confirmed by fraction collection and bioassay to be artifacts of freeze-thaw cycles.

He used capillary sieving separations of SD 5-protein complexes to develop a protein fingerprint of the dissociated virus, and this fingerprint was used to compare the profiles of structural and core proteins from viral batches. He also used sieving separations to profile restriction digests of the viral DNA to confirm the integrity of the viral genome. He compared the separations obtained with a conventional CE system with results obtained using an Agilent LabChip system (Agilent Technologies, Palo Alto, California). The LabChip system provided separations in 90 s, but they had lower resolution than the conventional system’s.

**Electroosmotic Flow:**

Taming the Beast

Dr. Jekyll and Mr. Hyde personify the two faces of electroendosmotic flow in electromigration separations. In CE, electroosmotic flow can transport analytes down the capillary and allow both anions and cations to be detected in the same analysis. In MEKC and capillary electrokinetic chromatography, electroosmotic flow is the pump that drives the mobile phase. However, variations in the magnitude of electroosmotic flow compromise the precision of migration times and peak areas. Variable electroosmotic flow arises from changes in the charge density on the inner surface of the capillary, and adsorption of sample components is the most common contributor to changing wall chemistry. Analysts have used various approaches — including adsorbed or bonded capillary coatings, buffer additives, and externally applied electric fields — to control or eliminate electroosmotic flow.

Roland Chevigné of Analis s.a. (Namur, Belgium) described a new dynamic coating system for controlling electroosmotic flow. This system is a double coating that is applied in two steps. In the first step, a polycationic polymer (the initiator) is introduced into the capillary and forms a stable coating by electrostatic association with silanol groups on the fused-silica surface. In the second step, a polyanionic polymer (the accelerator) is pumped into the capillary. This polymer associates with the initiator polymer and presents a negatively charged surface to the lumen of the capillary. The accelerator polymer is available in various background electrolytes of different pHs, which can be blended to...
achieve the desired pH for a particular application. The negative charge of the double coating is independent of pH, so the magnitude of electroosmotic flow is constant across a pH 2.5–9.3 range. This pH stability results in stable migration times from run to run and from capillary to capillary. Chevigné presented peptide and protein analysis applications of the double-coating system, and a user in the postpresentation discussion reported good results with basic drugs. The product is marketed in the United States by MicroSolv Technology Corp. (Long Branch, New Jersey) under the name CElixir.

Future Prospects
This symposium provided a forum for CE practitioners in the pharmaceutical industry to discuss applications of the technology and for prospective users to evaluate where CE would be useful in their environments. The fact that dozens of validated CE methods are now in use for small-molecule drugs and biopharmaceuticals attests to the usefulness of the technique. The third symposium in this series will be organized by Stacey Ma and Tony Chen of Genentech, Claudia Jochheim of Immunex, and SungAe Park of Amgen. The meeting will be held at the Hilton Boston Back Bay in Boston, Massachusetts, on 19–21 August 2001. For details about the meeting program or to submit an abstract, contact the California Separation Science Society, c/o Rhema Association Management, 156 South Spruce Avenue, Suite 207A, South San Francisco, CA 94080-4556, tel. (650) 876-0792, fax (650) 876-0793, WWW http://www.casss.org/cepharm.

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