

## **Table 4: iCIEF Applications and Challenges for Next-Gen Biopharma**

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### **Scope:**

Imaged Capillary Isoelectric Focusing (iCIEF) is an effective method for determining charge heterogeneity and isoelectric point and is used for product characterization, process monitoring, and product stability. It is used throughout the industry through all stages of development as a gold standard. New innovations have been made recently for iCIEF to bring novel applications and start a new chapter for iCIEF technology.

### **Questions for Discussion:**

1. What are the challenges in iCIEF applications?
2. What do users want to see improved in the current technology?
3. How does iCIEF help to develop next-gen biopharma such as AAV, mRNA, complex proteins, etc.?
4. How are novel applications of iCIEF such as fluorescence detection, preparative iCIEF, iCIEF-MS, etc. helping in the development of next-gen biopharma?

### **Discussion Notes:**

*What challenges exist in the iCIEF technology, and what needs to be addressed in the next generation of instrumentation?*

- iCIEF is used for QC, considered the golden standard for all stages of drug development
  - Challenges present due to chemicals or analytes
  - Profiles are not always consistent between stages of development
  - Method development takes time to develop a good profile
    - Reduce background noise, eliminate ghost peaks, remove bubbles
  - Baseline noise reduction
    - Shift from iCE3 > Maurice analysis allows the incorporation of fluorescence detection, leading to less worry about dirty samples
  - Main challenge is the chemicals used for sample prep
    - i.e., Urea: introduces denaturation
    - Ampholytes – have been issues with quality and lot variation
      - less resolution in high pH range, high pI molecules are harder to analyze robustly

- Urea: concern about denaturation
  - Changed to using Urea more as a last resort, need to think about what stage the samples are in and whether its needed
  - SimpleSol (proprietary ProteinSimple solubilizer) as an alternative to Urea
- Perspective 1: Always try and begin method development without Urea
  - General initial method development strategy: start with pH 3-10 carrier ampholyte, no urea and no pI markers – gives a baseline and shows where development focus needs to be
    - Only add urea if spikes due to aggregation are present
    - Start with 4M Urea and reduce if higher concentration is not needed; if still observing aggregation, need to increase concentration to 8M (no difference seen in product aggregation from the range of 4M to 8M)
    - Sucrose is an alternative to urea, but it will also deform proteins
- Aside: What is a native protein? Question to add perspective as to what is necessary to look at and attributes are necessary to qualify.
- No difference in urea saturation in the 4-8M range, only needed for samples with aggregation or other additives
- Perspective 2: Start with 2-10, covers a wide range of analytes, and determine if there is any need for stabilization
  - Sometimes lower than 4M urea will work if there is already a stabilizer as part of the matrix
- Platform methods – try and find a method that would be applicable for a wide range of pIs
  - Did not include Urea of the platform
  - Urea is hard to dissolve and takes time to react, so it needs time to sit with the sample
    - Cannot sit too long or it begins to degrade
- On-board mixing
  - Still need to be careful of urea
  - Urea needs to be made fresh
- Switch to glucose
- New paper out with a list of various solubilizers
- Formamide as an alternative
  - Protein is still not super happy (back to the question, What is a native protein?)
- Histology – use histo-structures to identify properties of the proteins and get an idea of protein stability
- CD (circular dichroism) – sensitive to small changes in secondary structure
  - Able to determine if changes are permanent or if the protein will return to a 'native state' stress conditions are applied
- Low concentration – add dye to help visualize

- Ampholytes – struggle for years (since the beginning of the introduction of icIEF to the industry)
  - Pharmalytes initially had no expiration date – manufacture stated that due to the fact they are small molecules, there was no change over time
  - Large lot to lot inconsistency in pharmalyte and pI markers
- AES (Advanced Electrophoresis Solutions) is very consistent ampholyte composition and pI markers
  - Do additional studies to ensure that all lots are comparable/reproducible
  - Focus on working on the next generation of technology
  - Customers are using them in gels, and use liters of ampholytes, so there is a high need for consistency in all lots
- AES recently developed ampholytes for fusion proteins, eliminating the need for servalyt and urea
- Servalyt vs Pharmalytes
  - Pharmalytes – sulfuric acid backbone contributes noise
  - Servalyt – introduces charge distribution
- AES ampholytes are closer related to pharmalytes with the reduction of noise due to their backbone
- Servalyt – sometimes needed, but generally used as a mixture with pharmalytes
  - Certain molecules need the addition of servalyt, but keep concentration low at 0.2%

*What does industry need to improve for current icIEF?*

- Lot to lot consistency of consumables
- Better quality control for the cartridge
  - Cartridges are expensive, customers want to extend the lifetime for as long as possible
- Perspective 1: icIEF needs the coating on the cartridge that degrades over time/number of injections
  - maintenance can affect cartridge lifetime
  - manually clean cartridge with water and store in water
- Perspective 2: icIEF cartridges are stable, just need to wash and store
  - issues arise due to crystal formation from buffer components
  - best SOP is to wash, dry and store dry
- Maurice platform – stores cartridges dry
  - Able to use cartridge cleaning
  - Recommend to purge if the cartridge has been stored for a long time
- AES Hydrophilic coating – eliminates need for methyl cellulose (MC) for method development, can work better for hydrophobic molecules
  - Requires better maintenance, drying every time after use
- MC possess an issue when there is a desire to couple to mass spec (MS)
- Elimination of MC does not affect the focusing/viscosity (hydrophilic coating)

- Is needed for FC cartridge – cartridge longevity is reduced to 0 if MC is not included in the matrix
  - MC does delay the separation due to the viscosity, but does not have an impact on the separation or peak shape
- Protein Simple Prep – flex instrument will eliminate need for MC due to the differences in mobilization
  - Chemical mobilization

*Hydrodynamic mobilization can reduce mobilization time and potential shift of peaks.*

- AES: Pressure elution of the focused molecules – pushing the components into a smaller ID tube for fractionation
  - Allows for broadening of peaks to generate further separation of the components

*Improvement of icIEF – previously only focused on pI, now focused on fractionation, Fluorescence, coupled directly to MS*

- Perspective 1: Prefer fractionation and then transfer to MS rather than direct coupling
  - MS does not need to the high sample load
- Perspective 2: Direct couple should be better due to the ability to have full automation of icIEF analysis and immediate injection onto an MS system
- Perspective 3: Depends on need – QC vs. research, and how much analysis will be needed
- Smaller bore for MS, larger for fractionation
  - Is it still native in the fractionated states? (What is native?)
- The less additives, the higher the chance the product is as is (native), creates more honest comparisons
  - Don't know if protein is always a stable environment for ideal protein folding
    - Need to add salt and other components to ensure that the protein is in the proper folded state
- cIEF – separate cells and viruses, can handle large products and proteins intact

*What makes proteins change? Heating, chemicals, organics – most controversial is urea*

- Native MS – try to keep protein in the native environment
  - Additional processing can impact protein structure
- Study the effects of different treatments and see what comes out
  - i.e., more acidic peaks and what it means
- Low concentration – i.e., mRNA and other small molecules
  - Will be applicable to the next generation of therapeutics
- People are already using icIEF for mRNA, fusion proteins
- Addition of fluorescence allows for low concentration analysis

- How to measure samples from patient blood in a native state
  - icIEF can do Erythropoietin (EPO) – for red blood to get more oxygen

*What do we think is the future of the technique – where does it go?*

- Robotization – develop sample prep that can be done on a robotic machine/liquid handler will prepare the samples
  - Urea, protein, all components added to the liquid handler
  - Samples are prepared at room temperature
    - Sometimes see aggregation with sticky proteins – adjusted urea to reduce the aggregation
  - Determining factor of robot use – sample load; not necessarily needed for 12 samples, but is needed for 50 samples
- Robotization example – testing needs standard curves run on plates, previously done manually, compared linearity results to automated prep with the Hamilton
  - Human: 5% error
  - Hamilton: 2% error
- Robots can have issues when there are time dependent chemical reactions
- Robots can increase reproducibility of analysis
- Cost-benefit analysis on whether the speed efficiency is more important or the reproducibility
- Analysts can introduce bubbles from pipetting too fast