ISSN 2573-1815 · SSC 6 (5) 2023 · Vol. 6 · No. 5 · May 2023

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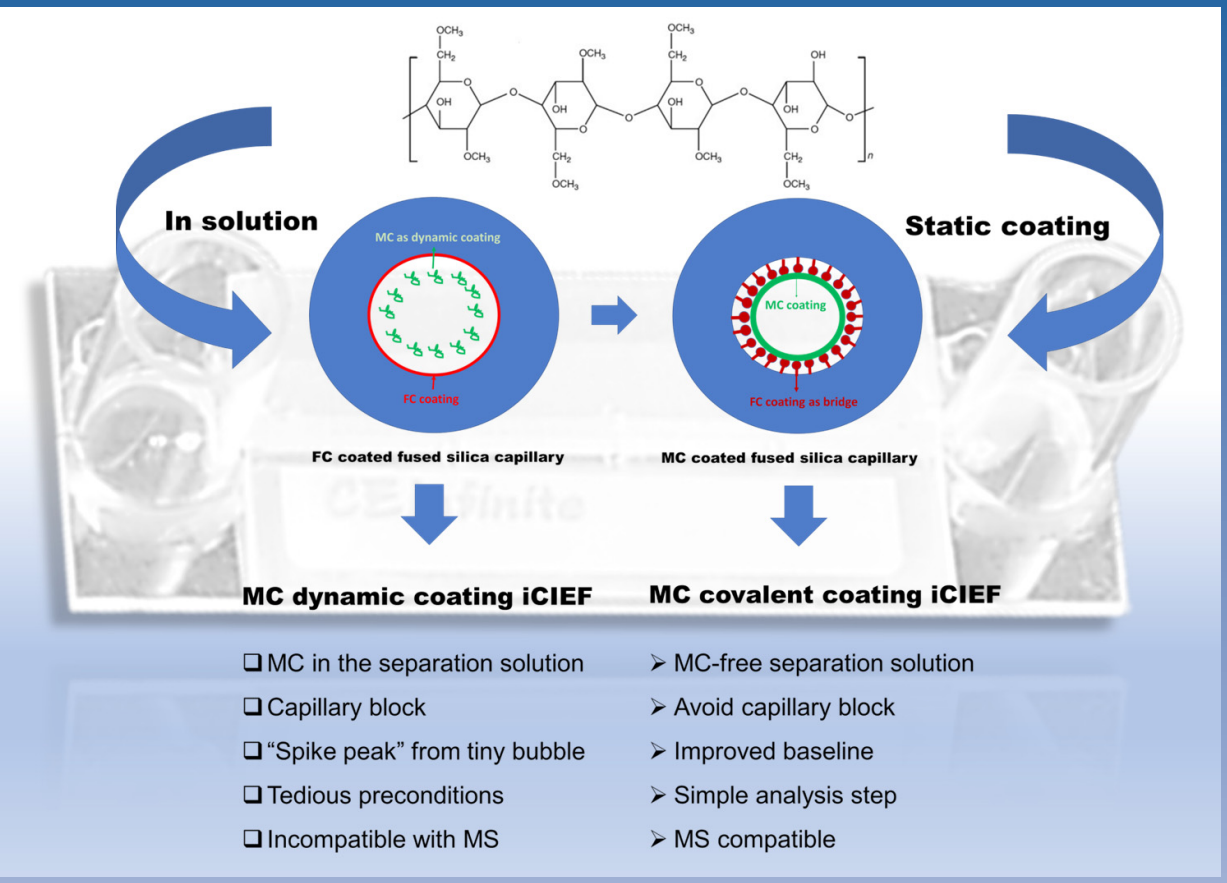

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Received: 19 December 2022 | Revised: 4 February 2023 | Accepted: 6 February 2023

DOI: 10.1002/sscp.202200160

RESEARCH ARTICLE

Imaged capillary isoelectric focusing employing fluorocarbon and methylcellulose coated fused silica capillary for characterization of charge heterogeneity of protein biopharmaceuticals

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Identifying and characterizing charge heterogeneity is essential for the successful development and production of biopharmaceuticals. Imaged capillary isoelectric focusing technology based on isoelectric point differentiation has been becoming the gold standard of quality and manufactory process control in the biopharmaceutical industry due to its high-resolution characterization of protein charge variants with high throughput. Fluorocarbon-coated capillaries are widely used in the imaged capillary isoelectric focusing to suppress the electroosmotic flow and minimize the protein adsorption onto the fused silica capillary inner surface. Additionally, polymers such as methylcellulose are usually added to the sample solution to serve as a dynamic capillary coating that improves the peak shape and resolution during the imaged capillary isoelectric focusing separation, especially for complex proteins. However, the addition of methylcellulose tends to result in tedious operation, "spike peaks" from bubble generation, and frequent capillary blockage during the imaged capillary isoelectric focusing separation. In addition, methylcellulose is not compatible with mass spectrometry and easily produces the contamination of mass spectrometry ion source when carrying out imaged capillary isoelectric focusing—mass spectrometry direct coupling. Recently, a new imaged capillary isoelectric focusing method was developed employing a methylcellulose-coated capillary cartridge, to avoid the addition of methylcellulose which is then present throughout the whole analysis. When applied to protein drug characterization the established imaged capillary isoelectric focusing method demonstrated high repeatability, stable coatings, outstanding separation efficiency, and excellent isoelectric point differentiation. In addition, we compared imaged capillary isoelectric focusing separation using the methylcellulose-coated capillary with that utilizing the routinely coated capillary such as fluorocarbon, illustrating that methylcellulose

Article Related Abbreviations: AD, acrylamide; ADC, antibody-drug conjugate; FC, fluorocarbon; iCIEF, imaged capillary isoelectric focusing; mAbs, monoclonal antibodies; MC, methylcellulose.

https://doi.org/10.1002/sscp.202200160

coating provided consistent results and could be seamlessly integrated into an existing drug discovery process. Finally, the methylcellulose-coated fused silica capillary was applied to imaged capillary isoelectric focusing—mass spectrometry for characterizing protein charge variants allowing reliable identification of mass spectrometry after imaged capillary isoelectric focusing separation. This can greatly simplify the operation steps and prevent the contamination of mass spectrometry ion source that often results from using routinely coated capillaries ultimately making this an essential innovation of imaged capillary isoelectric focusing—mass spectrometry that greatly improves the imaged capillary isoelectric focusing compatibility with mass spectrometry.

KEYWORDS
antibody, coated capillary, fluorocarbon, imaged capillary isoelectric focusing, mass spectrometry

1 | INTRODUCTION

Protein charge variants arise from a variety of mechanisms including chemical degradation, post-translational modification, and production conditions during the manufacturing process [1–4]. These alterations can cause changes in the pI of a protein which can affect the safety and efficacy of a protein drug [5, 6]. Therefore, understanding and characterizing the charge heterogeneity of protein drugs is a critical step in the commercial and clinical production of biotherapeutics. For this purpose, Imaged capillary isoelectric focusing (iCIEF) technology based on pI differentiation is becoming the gold standard across the pharmaceutical industry [7, 8]. Recently, more complex proteins including antibody-drug conjugates (ADCs), bi-specific antibodies, and fusion proteins have attracted great attention as potential therapeutic candidates [9–11]. This has required advancements in iCIEF technology to maintain a highly repeatable, high-resolution strategy for an optimal drug discovery workflow.

In fact, reproducible iCIEF separation of larger biomolecules using bare fused silica capillaries is rare due to protein adsorption [12]. Many proteins have large localized regions of positive charge that are electrostatically attracted to the negatively charged silanol groups at the capillary inner surface. Additional adsorption can be caused by hydrophobic and hydrogen bonding interactions. In iCIEF separation, the electroosmotic flow (EOF) generated by this adsorption along the capillary can seriously impend the complete sorting of proteins according to their pIs. Hence, coating technologies using hydrophobic fluorocarbon (FC) or acrylamide (AD) chemically linked to the capillary wall (static), have been widely used to suppress EOF for more efficient iCIEF protein separation [13–15]. Hydroxyethylcellulose and hydroxypropylcellulose were also reported for both dynamic and covalent coatings of the fused silica capillary in CIEF [16, 17]. In commercial iCIEF analysis, FC-coated capillaries have been proven to be the most stable and popular option for protein separation. Although such coated capillaries can effectively decrease protein adsorption onto the fused silica capillary's inner surface, the residual negatively charged silanol groups, which cannot be fully shielded by FC coatings, tend to adsorb the proteins, resulting in low separation efficiency and poor repeatability. Thus, neutral polymers such as methylcellulose (MC) are usually added to the protein sample solution containing proteins and ampholytes to create a dynamic coating as shown in Figure 1. This process further eliminates interactions between proteins and capillary inner surface resulting from potential regions of exposed silanol region after the static coating by FC and preserves the repeatability and efficiency of protein separation in iCIEF. Unfortunately, the addition of MC tends to result in tedious operation, "spike peaks" generation associated with high solution viscosity, and even capillary blockage during the iCIEF separation. The required pre-rinsing of the coated capillary with MC solution also results in low analysis throughput. Moreover, HRMS is currently the most powerful means of qualitative protein analysis [18, 19], but MC is not compatible with MS and easily produces the contamination of MS ion source, which accordingly hinders the applications by iCIEF-MS direct coupling.

In our work, an MC permanently coated capillary based on bilayer polymerizations was developed for iCIEF characterization of protein charge variants. This study aims to simplify the operation steps in the iCIEF analysis and prevent the contamination of the MS ion source resulting from using routinely coated capillary requiring pre-rinsing with MC solution before sample running. The

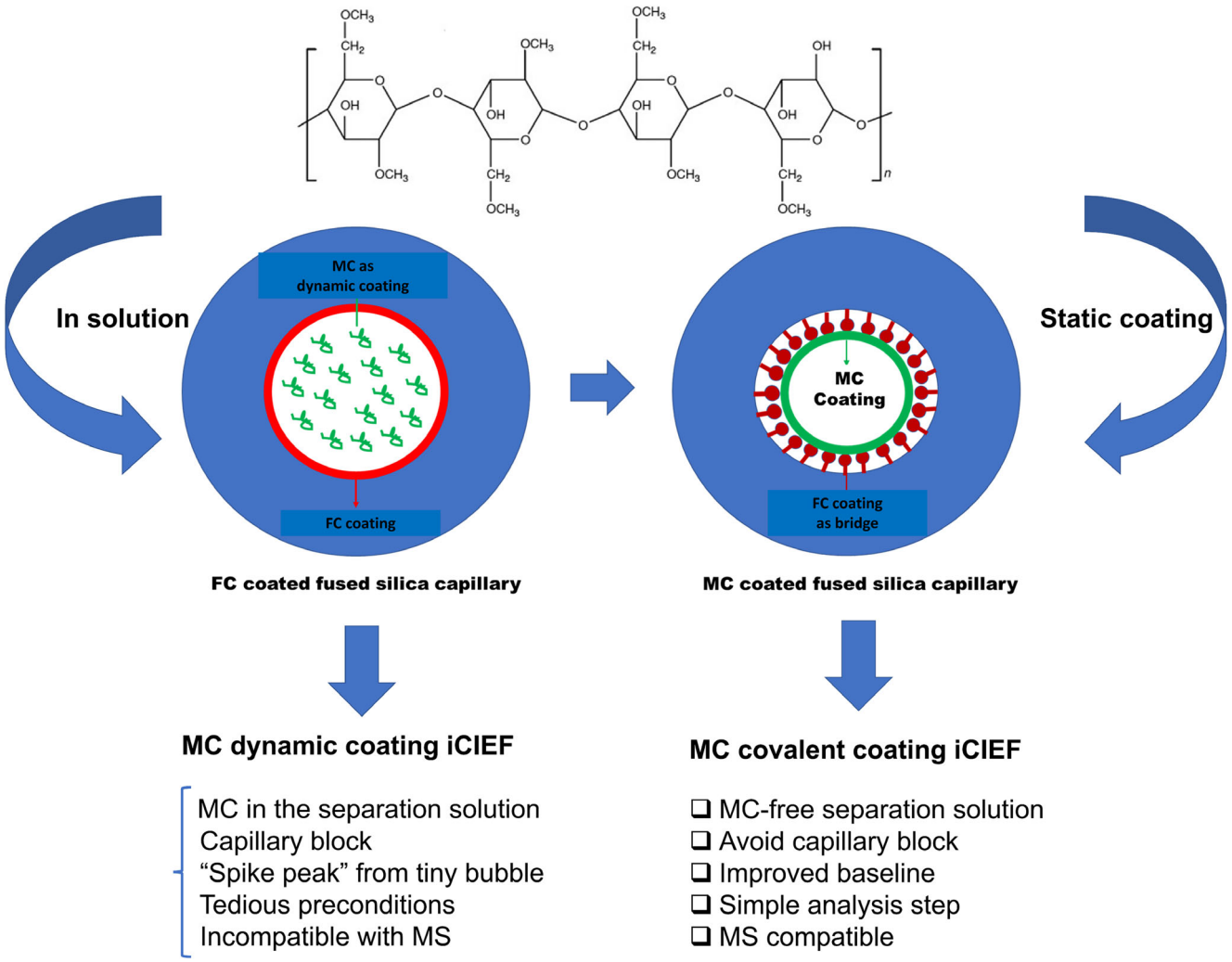

FIGURE 1 Advantages of methylcellulose (MC) static coating in imaged capillary isoelectric focusing (iCIEF) as compared to MC dynamic coating.

developed method effectively avoided the addition of MC as a dynamic coating and was free from pre-conditioning with MC solution before the iCIEF separation. Excellent long-term stability of MC-coated capillary was achieved and the iCIEF behaviors of diverse protein drugs including monoclonal antibodies (mAbs), ADC, bi-specific antibodies, and fusion proteins were compared with the use of FC-coated and MC-coated capillaries to ensure the methodological consistency. Finally, the MC-coated fused silica capillary was applied to iCIEF-MS for characterizing protein charge variants with reliable MS identification after iCIEF separation. Ultimately, the methods based on MC coatings, outlined herein, are robust, highly efficient, and have high throughput for iCIEF quality control and iCIEF-MS direct coupling.

2 | MATERIALS AND METHODS

2.1 | Chemicals

AESlyte carrier ampholytes and all pI markers were obtained from Advanced Electrophoresis Solutions Ltd (AES, Cambridge, Ontario, Canada). Pharmalytes carrier ampholytes were purchased from Cytiva Ltd. Monoclonal antibody NISTmAb was purchased from Millipore Sigma (cat. no. NIST8671). Other protein drugs including mAbs (avastin-bevacizumab), mAb-AT-1, ADC-AT-1, bi-specific Ab-AT-1, and fusion protein-AT-1 employed in this study were kindly donated from Thermo Fisher Scientific (China). MS-grade ACN, formic acid, formamide, and MC were purchased from Fisher Scientific (Hampton, NH).

2.2 | Solutions for iCIEF

For iCIEF-UV and iCIEF-MS, the sample solutions for protein drugs employed in this study were listed in Table 1.

2.3 | Imaged capillary isoelectric focusing

CEInfinite iCIEF (Advanced Electrophoresis Solutions Ltd, Cambridge, Canada) was employed in this study. By utilizing bi-layer polymerization, MC-coated capillary cartridges developed are proprietary by AES as shown in Figure 1 and the bridge coatings of FC as the first polymerization layer and following MC coatings. 100 μm id MC-coated (AES, cat. no. CP00501) and 100 μm id FC-coated (AES, cat. no. CP00201) capillaries are utilized for iCIEF-UV analysis. 200 μm id MC-coated (AES, cat. no. CP00503), 200 μm id AD coated capillaries (AES, cat. no. CP00303), and micro-tee integrated (AES, cat. no. CP00303M) were used for iCIEF-MS. All coated capillaries had an od of 360 μm. All these WCID (Whole Column Imaging Detection) cartridges have a 5 cm long separation capillary, and a 50 μm id transfer capillary is assembled for both iCIEF-MS. The 200 μm AD coated iCIEF-MS capillary used for iCIEF-MS includes a quartz union (works as a micro-tee), connecting the make-up solution and transfer capillary to the ion source of MS. Both the make-up solution capillary and transfer capillary have a 100 μm id.

As for iCIEF-UV analysis, the focus was 1 min at 1000 V, 1 min at 2000 V, and 10 min at 3000 V. As for iCIEF-MS, the focus was 1 min at 1000 V, 1 min at 2000 V, and 10 min at 3000 V, and 3000 V during mobilization; the mobilization speed was 50 or 100nL/min with water containing 0.1% v/v formic acid, across the separation capillary, and 5 μl/min make up solution (water:ACN = 1:1, v/v, containing 1% v/v formic acid) added through a micro tee. Mobilization time was 15 min.

2.4 | High-resolution MS

A Thermo Q Exactive Plus mass spectrometer with a Biopharma option equipped with an Ion Max ESI Ion Source with a 34-gauge needle (Thermo Fisher Scientific, Bremen, Germany) was used for mass measurement. The spray voltage: 3.6 kV, the sheath gas: 20 L/min, the auxiliary gas: 5 L/min, the S-lens RF: 70 eV, the capillary temp: 275°C, the resolution: 35 000@m/z 200, the scan range of precursor ion: 2000–8000 m/z, and the maximum injection time: 200 ms.

TABLE 1 Composition of the sample solution for imaged capillary isoelectric focusing (iCIEF) separation of various protein drugs.

a) NISTmAb		
Composition	**Concentration**	**Volume (μl)**
Urea	2 M	50.0
Pharmalyte 3-10	1%	2.0
Pharmalyte 8-10.5	3%	6.0
pI Marker 8.18	0.5%	1.0
pI Marker 9.77	0.5%	1.0
NISTmAb	0.4 mg/ml	8.0
Nano Pure Water	/	132.0
b) Avastin-Bevacizumab		
Component	**Concentration**	**Volume (μl)**
Urea	2 M	50.0
Pharmalyte 3-10	4%	8.0
pI Marker 6.14	0.5%	1.0
pI Marker 9.33	0.5%	1.0
Avastin-Bevacizumab	0.5 mg/ml	10.0
Nano Pure Water	/	130.0
c) Fusion Protein AT-1		
Composition	**Concentration**	**Volume (μl)**
AESlyte UH 4-10	2%	4.0
Iminodiacetic acid (100 mM)	10 mM	20.0
pI Marker 3.38	0.45%	0.9
pI Marker 7.05	0.05%	0.1
Fusion Protein AT-1	0.25 mg/ml	1.95
Nano Pure Water	/	173.05
d)Bi-Specific Antibody AT-1		
Component	**Concentration**	**Volume (μl)**
Urea	1 M	40.0
AESlyte HR 9-12	4%	8.0
pI Marker 8.18	0.25%	0.5
pI Marker 10.10	0.165%	0.33
Bi-Specific Antibody AT-1	0.3 mg/ml	2.01
Nano Pure Water	/	149.16
e) ADC-AT-1		
Component	**Concentration**	**Volume (μl)**
AESlyte HR 3-10	1%	2.0
AESlyte 8-10.5	3%	6.0
pI Marker 8.71	0.5%	1.0
pI Marker 9.77	0.5%	1.0
ADC-AT-1	0.2 mg/ml	8.0
Nano Pure Water	/	182.0
f) mAb-AT-1		
Component	**Concentration**	**Volume (μl)**
AESlyte HR 7-8	2%	4.0
ADC-AT-1	1 mg/ml	40.0
Nano Pure Water	/	156.0

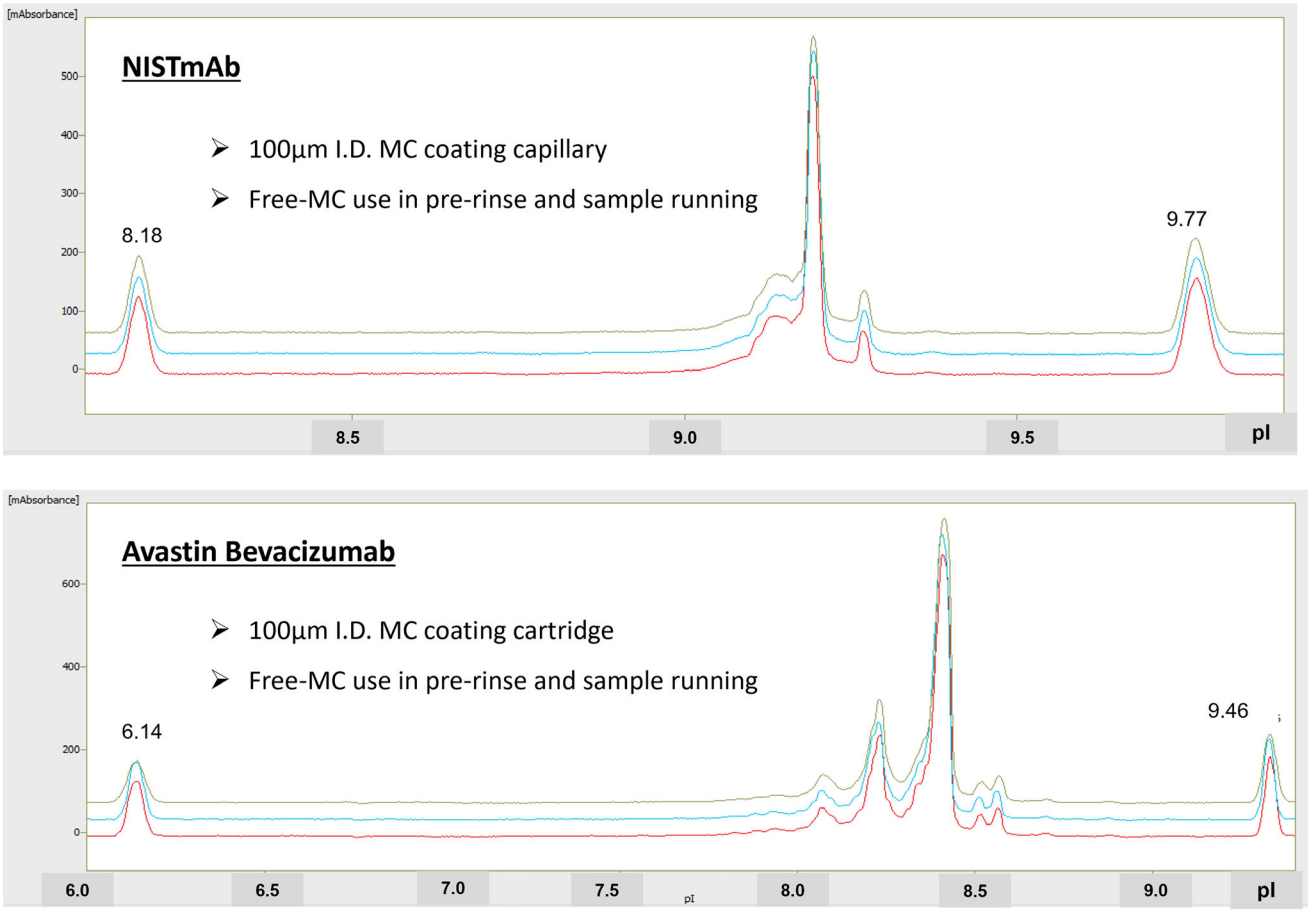

FIGURE 2 Imaged capillary isoelectric focusing (iCIEF)-UV profiles using methylcellulose (MC)-coated capillary for NISTmAb and avastin bevacizumab without MC as additive (n = 3). For composition of the sample solution, see Table 1. The focusing was 1 min at 1000 V, 1 min at 2000 V and 10 min at 3000 V.

Biopharma Finder (BPF 5.0) from Thermo Fisher was used for the data analysis including intact protein deconvolution and peptide mapping.

3 | RESULTS AND DISCUSSION

In this work, a strategy of bilayer polymerizations was developed for the static coating of MC in the capillary, to achieve the high-efficient characterization for protein charge variants. The first layer of FC coating onto the capillary inner surface was achieved as a bridge that constructed the second static coating layer with MC as illustrated in Figure 1. The innovative MC-coated capillary successfully avoids addition of MC as a dynamic coating and omits pre-condition of MC solution before the iCIEF separation.

The unique MC-coated capillary was applied to characterize the charge variants of therapeutic mAb including NISTmAb and avastin-bevacizumab as shown in Figure 2, demonstrating excellent separation resolution with good repeatability. Without the use of solution MC, the experimental workflow is simplified ultimately allowing a much higher- throughput of analysis.

3.1 | Stability of MC-coated capillary

As seen in Figure 3, the MC-coated capillary demonstrated excellent long-term repeatability with the consistent iCIEF profile by employing NISTmAb as targeted protein in the consecutive 80 runs. Supporting Information 1 demonstrated outstandingly repeatable peak areas of charge variants (RSD < 5.0%).

3.2 | Comparison of iCIEF separation using FC- and MC-coated capillaries

The study indicated that the FC- and MC-coated capillaries exhibited comparable iCIEF separation with good resolution for NISTmAb and avastin bevacizumab in Supporting

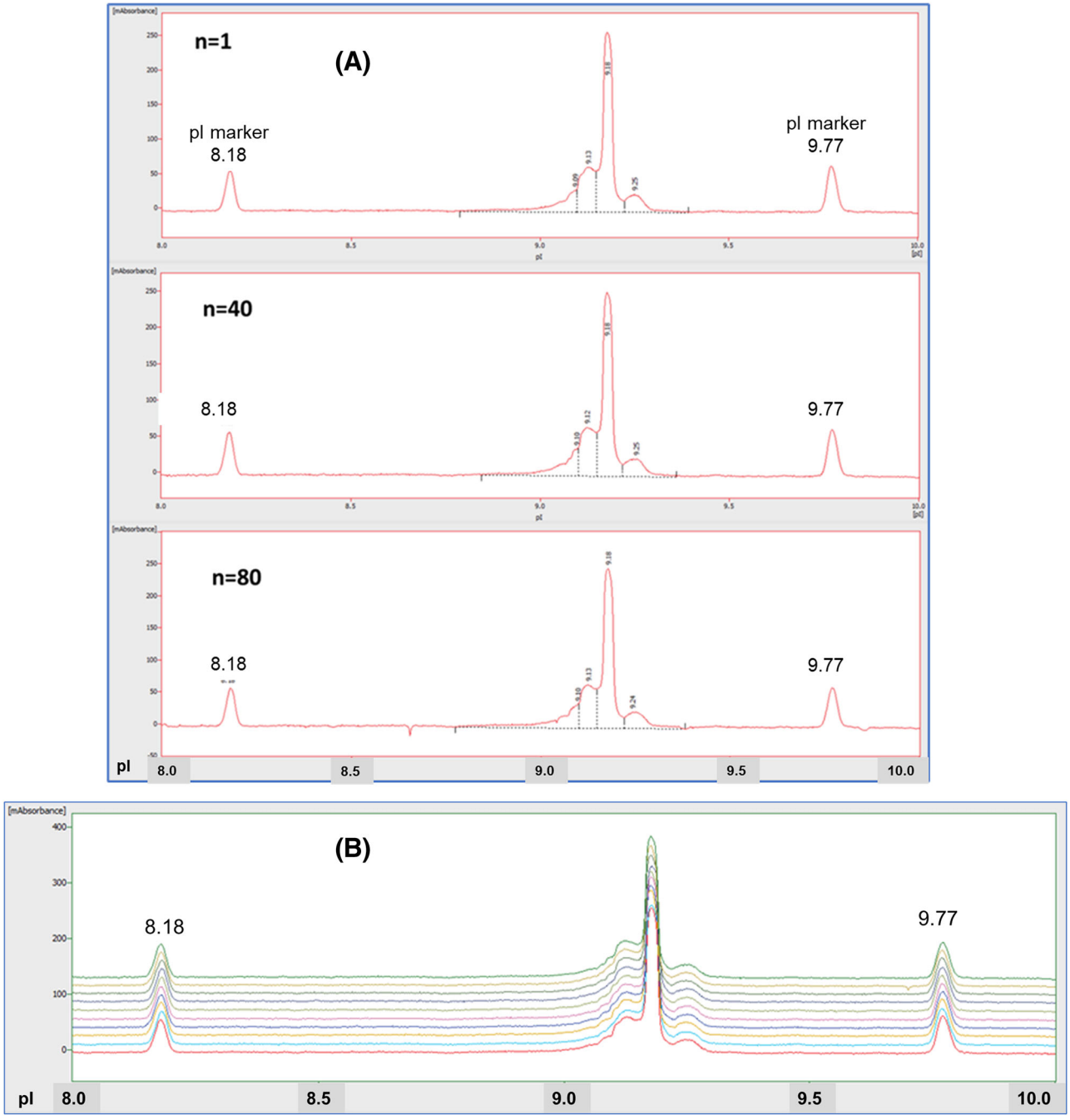

FIGURE 3 Imaged capillary isoelectric focusing (iCIEF) repeatability of NISTmAb using methylcellulose (MC)-coated capillary. (A) consecutive 80 runs; (B) repeatability of ICIEF analysis ($n = 10$). Long lifetime of coating was demonstrated. For composition of the sample solution, see Table 1. The focusing was 1 min at 1000 V, 1 min at 2000 V and 10 min at 3000 V.

Information 2. The FC-coated capillary utilized sample solutions containing 0.35% v/v MC and pre-conditioning with MC was performed before each run. Meanwhile, the MC-coated capillary is completely free from using MC solution during the whole analysis. The iCIEF profiles obtained were consistent for both FC- and MC-coated capillaries illustrating that this coating can be easily integrated into a pre-existing method development process.

3.3 | MC-coated capillary for complex proteins

Complex proteins including fusion protein, bi-specific Ab, and ADC have attracted significant attention in the biopharmaceutical industry [9–11] but characterizing their charge heterogeneity is challenging due to their complex protein compositions. As illustrated in Supporting

Information 3, the MC-coated capillary developed was employed for various types of complex protein therapeutics with excellent pI differentiation for protein isomers. It was observed that the peaks of the fusion protein were acidic, located in the narrow range of pI 4.0-6.5 with major three peak groups (pI 4.0–5.1, pI 5.1–5.5, and pI 5.5–6.5) (Supporting Information 3A). The Bi-specific Ab demonstrated rather basic pIs—around 9.5 for the main protein and its four charge variants (Supporting Information 3B) and finally, the ADC studied also indicated basic properties with pIs in the range of 8.7–9.2 for major protein peaks (Supporting Information 3C). This represented the wide applicability of MC-coated capillary in a diverse of therapeutic proteins.

3.4 | iCIEF-MS for characterizing protein charge variants using MC-coated capillary

In this study, the developed iCIEF-HRMS system and patented capillary cartridges [20–22] eliminate the need for chemical migration when coupled to online mass spectrometry as shown in Supporting Information 4A. Furthermore, using proprietary capillary-coated cartridges and separation solutions during iCIEF separations greatly reduces the need for polymers and urea. It enables the isolated protein charge variants to be directly used for high-sensitivity MS characterization, thus retaining the excellent separation resolution of iCIEF for mass spectrometry analysis. The seamless interface to MS based on micro-fluidity prevents sample loss enhancing the sensitivity of MS detection of separated proteins. The constructed system requires no special modifications to the ionization source and can be directly connected to the mass spectrometers from the different leading mass spectrometry brands.

After proteins' focusing along the separation capillary is completed, water containing 0.1% v/v formic acid functions as a mobilization solvent from the syringe pump to drive the focused protein bands out of the separation capillary towards the MS ion source at 50–200 nl/min flowing rate depending on selected separation capillary id (typically 50 nl/min for 200 μm id capillary; and 160 nl/min for 320 μm id capillary). Sheath liquid or make-up solution (water:ACN = 1:1 v/v, containing 1% v/v formic acid) helps the effluents directly into MS ion source though a seamless interface. The whole process is automatic and user-friendly.

To demonstrate the high accuracy of iCIEF-MS for protein M_r measurement, we validated that the deconvoluted M_r (molecular mass) of the main component of bevacizumab' was consistent by HPLC–MS and ICIEF-MS (Supporting Information 4B). This guarantees the methodological accuracy of ICIEF-MS coupling in the characterization of intact protein charge variants. Furthermore, an intact NISTmAb was utilized for the stability evaluation of the iCIEF-MS methodology, to achieve good repeatability (Supporting Information 4C). The total ion CIEF-gram demonstrates highly repeatable results ($n = 3$) in terms of elution times and measured M_r due to coated capillary stability and the compatibility of ampholytes with MS contributes to high sensitivity in MS detection. This warrants the applicability of iCIEF-MS established in characterizing diverse of charge variants of protein drugs.

As mentioned previously, the developed iCIEF-MS method employed an MC-coated separation cartridge, to simplify operation steps and prevent contamination of the MS ion source that can result from using a traditional capillary pre-rinsed with MC solution. As shown in Figure 4, the MC-coated fused silica capillary (Figure 4A) achieved comparable results to an AD coated capillary (Figure 4B) while avoiding the use of MC during the sample running of NISTmAb. This is an outstanding advantage of iCIEF-MS to greatly improve the compatibility with MS meanwhile obtaining comparable iCIEF-MS behaviors. It is worthy of being mentioned that the migration times of protein change variants on the MC-coated capillary are a little longer than those with the use of AD coated capillary, which supported the lower EOF level resulting from a static MC coating. To account for this, the flowing rate of mobilization solvent from syringe pump can be flexibly tuned from 50–100 nl/min to optimize the iCIEF-MS analytical time.

As illustrated in Supporting Information 5, using a very narrow pH range ampholyte (pH 7–8), mAb-AT-1 with its four charge variants was profiled by iCIEF-MS with good sensitivity. Lastly, the developed iCIEF-MS platform was further extended to characterize the charge variant of a more complex protein (bi-specific Ab) (Supporting Information 6). This provided fast and accurate fingerprinting of the proteins' charge heterogeneity including a main component, two acidic variants, and two basic variants. Furthermore, for even the smallest peaks (A2/B2) an MS spectrum with good S/N was obtained illustrating the high sensitivity of this system in Supporting Information 6B.

4 | CONCLUDING REMARKS

In this work, an innovative iCIEF method was developed employing an MC-coated capillary cartridge, to avoid the need for MC in the whole process of analysis. The innovative iCIEF method was utilized for protein drug characterization, which demonstrated high repeatability, outstanding coating stability, excellent separation efficiency, and robust pI differentiation for a diverse set of

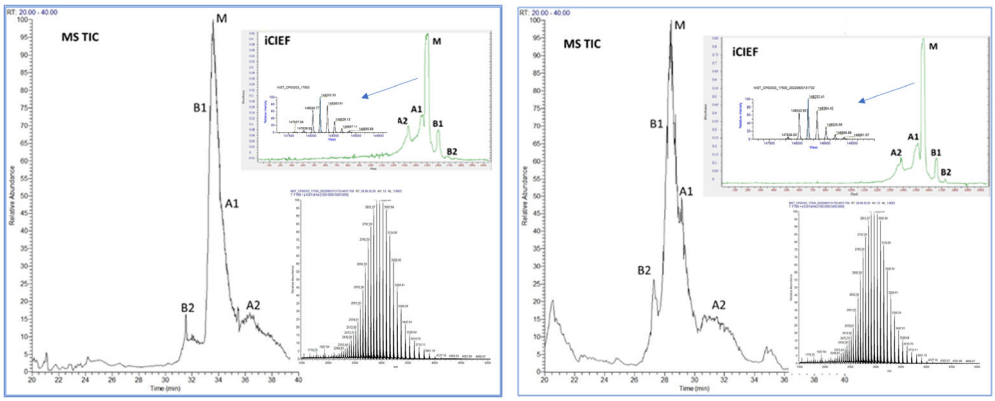

FIGURE 4 Imaged capillary isoelectric focusing (iCIEF)-MS of NISTmAb: (A) Methylcellulose (MC)-coated capillary; (B) Acrylamide (AD)-coated capillary. The comparable results using both coated capillaries were obtained. The focusing was 1 min at 1000 V, 1 min at 2000 V and 10 min at 3000 V, and 3000 V during mobilisation; the mobilisation speed was 50 or 100 nl/min with water containing 0.1% v/v formic acid, across the separation capillary, and 5 μl/min make up solution (water:ACN = 1:1, v/v, containing 1% v/v formic acid) added through a micro tee. Mobilisation time was 15 min.

protein therapeutics including mAb, ADC, fusion protein, and bi-specific mAb. Moreover, the iCIEF separation using the MC-coated capillary can be compared with that of the routinely coated capillary such as FC, to ensure consistency during the whole process of drug discovery. Following validation, the MC-coated capillary was used for the iCIEF-MS characterization of protein charge variants for a diverse set of protein therapeutics. The removal of MC from the experimental workflow greatly improved the compatibility with MS; eliminating the required pre-rinse with MC allowed a more automated and user-friendly operation. This combined with the reliable and rapid identification of MS peaks after iCIEF separation proves that the developed iCIEF-MS method outlined here is a cutting-edge technology in iCIEF-MS development.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from Federal Economic Development Agency for Southern Ontario. We thank Aotian Bo from Laurel Heights Secondary School (Waterloo, Ontario, Canada) as an Intern at AES Ltd for his work in the sample preparation and iCIEF method development. And technical support from Thermo Fisher Scientific is highly appreciated.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kwok T, Chan SL, Shi J, Zhou M, Schaefer A, Bo T, Li V, Huang T, Chen T. Imaged capillary isoelectric focusing employing fluorocarbon and methylcellulose coated fused silica capillary for characterization of charge heterogeneity of protein biopharmaceuticals. Sep Sci plus. 2023;2200160.
https://doi.org/10.1002/sscp.202200160