RESEARCH NOTE

Greening Capillary Electrophoresis, a promising sprout of Separation Science toward sustainability

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Received: 20/01/2020

Accepted (Online first): 30/04/2020

Vol./Issue/Year: 1(1), 2020

Competing interests: Author(s) stated no compete of interest.

Grant information: The author is grateful for the support of EFOP-3.6.3-VEKOP-16-2017-00009 co-financed by EU and the European Social Found.

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How to cite: Szarka, M. "Greening Capillary Electrophoresis, a promising sprout of Separation Science toward sustainability" *DRC Sustainable Future* 2020, 1(1): 60-65, DOI: 10.37281/DRCSF/1.1.8

ABSTRACT

As a result of miniaturization new avenues were open toward customizing, improving and rendering separation science more affordable and available to any laboratory worldwide. One of the best resolving liquid separation techniques that still benefits from miniaturization is capillary electrophoresis (CE), where analytes are separated by their hydrodynamic volume to charge ratio. The theory of CE was introduced almost one hundred years ago, but became popular in the 1970s, yielding by 2010 over 1000 papers produced yearly. This progress triggered sample preparation optimization efforts, which led to significant reduction of required chemicals for analysis and the decrease of overall sample processing times. Consequently, CE can be considered as a sustainable technique in the field of liquid phase separation science. In this paper a custom made, cheap capillary electrophoresis unit with LED induced fluorescent (LedIF) imaging detection was used to demonstrate applicability of modern electronics, consumer products, and 3D printing in generating scientific results, while keeping sustainability in mind. Samples were chosen according to the observed trends of the past decade, namely from biotherapeutics industry. Its golden standard, immunoglobulin G N-glycans were enzymatically digested and the released complex type oligosaccharides were labeled with charged fluorophore, according to one of the most advanced and optimized protocols. Results were compared to separation runs performed on a high quality commercially available instrument, used as the control. Results disclosed in this paper should not be subjected to direct quantitative comparison, but should be rather taken as a technical demonstration of the capabilities of current and future technology, which can be implemented and merged with existing solutions in a sustainable manner.

Keywords: sustainability, capillary electrophoresis LED induced fluorescence, N-glycans, IgG

Abbreviations: CE: capillary electrophoresis, LED: Light emitting diode, LIF: Laser induced fluorescence, LedIF: light emitting diode induced fluorescence, ID: inner diameter, BGE: background electrolyte, mAb: monoclonal antibody, Man1: Mannose, GlcNAc: N-acetylglucosamine, Asn: Asparagine, Ser: Serine, APTS: 8-aminopyrene-1,3,6-trisulfonic acid, GU: Glucose Unit, PMT: Photomultiplier tube, CMOS: Complementary metal-oxide-semiconductor, CCD: Charge-coupled device, PNGase F: Peptide:N-glycosidase F, THF: Tetrahydrofuran, HPLC: High-performance liquid chromatography, hIgG1: human Immunoglobulin G type 1, N-CHO: N-linked carbohydrate separation gel buffer

1. Introduction

Capillary zone electrophoresis (Jorgenson and Lukacs, 1983) is considered one easily adaptable liquid separation analytical techniques. It supports sustainability, since it requires extremely low amounts of reagents and laboratory consumables. In addition, the method has low power requirement and not only provides high sensitivity analytics, but also has the potential to reduce chemical, instrumental, and overall carbon footprint of separation science laboratories. The method is based on the differential electromigration of analytes in submillimeter diameter capillaries, filled with low conductivity buffer, difference arising from their hydrodynamic volume-to-charge ratio. Driving force of component migration is the high voltage applied to a narrow silica capillary (100-1000 V/cm) filled with low conductivity background electrolyte (BGE). Required BGE volumes to fill the capillary are usually between 0.1-1 μ L depending on the total length and the inner diameter (ID) of the capillary used for the separation. The reduced BGE need documents the eco-friendliness of the technique.

Over the past decade, N-glycans have become an important target molecules of this separation technique (Guttman, 1996), mainly as a result of the increased biomarker research and the emerging biotherapeutics industry (Lu *et al.*, 2018). The purpose of N-glycan analysis of therapeutic glycoproteins arises from the blockbuster monoclonal antibody drugs (mAb) and their biosimilars. Please note that biosimilars need to be proven similar to the original products for avoiding efficacy and safety concerns (Duivelshof, *et al.* 2019 ; Borza *et al.* 2018). Among the main contributors to immune-activity of these proteins are their N-glycan moiety, which demonstrate clinical applicability. N-glycosylation is a co/post-translational modification, where carbohydrates are linked to the nitrogen atom of asparagine amino acids present in the polypeptide chain, where there is a consensus sequence of asparagine-X-serine or threonine. X can be any amino acid apart from proline. N-glycans comprise a core trimannosyl chitobiose structure (Man1-6 (Man1-3)Man1-4GlcNAc1-4GlcNAc1-Asn-X-Ser), usually 1-5 % by weight of a glycoprotein, like Immunoglobulin G (De Leoz *et al.* 2020; and Szigeti *et al.* 2016). The analysis requires high performance approaches for their separation and detection as well (Bodnar *et al.*, 2016;

and Szigeti, M., *et al.*, 2016). Therefore, the starting procedure for CE based analysis of N-glycans is the selective cleavage of the asparagine-linked glycans from the glycoproteins by specific endoglycosidase enzymes, such as PNGase F. Then, labeling of the released oligosaccharides is done at their reducing end with a charged fluorophore, usually 8-aminopyrene-1,3,6-trisulfonic acid (APTS). As a next step the clean-up is performed with a microcolumn or by means of magnetic bead-based methods. The remaining (?) glycans labeled with charged fluorophore are ready to be separated by capillary electrophoresis equipped with laser or LED induced fluorescent detection (LIF or LedIF) (Ruhaak *et al.*, 2010).

Over recent years several improvements have been made to the analytical procedure, which translate into over 10x reduction in overall preparation time (Szigeti, M. and A. Guttman, 2017), less and less pollutant reagents used for labeling, cleanup or rinsing procedures, just to name a few (Kovacs *et al.*, 2016; and Kovacs *et al.*, 2017). In general, a volume of ~50 mL reagent – that involves all liquid handling during sample preparation – are enough for the preparation and processing of more than 100 samples, clearly supporting sustainability owing to newly developed commercial kits, such as the Fast Glycan kit by SCIEX.

Injecting nanoliters of sample into the capillary demonstrates convincingly how insignificant the environmental burden can be in laboratories adopting CE-LIF technology. Migration time of APTS-labeled carbohydrates serve for the calculation of glucose unit (GU) values to provide database-facilitated structural elucidation (Jarvas *et al.*, 2015). This calculation generally requires consecutive runs of APTS-labeled maltooligosaccharide ladder after each labeled N-glycan sample separation. Recently, this time consuming procedure was eliminated by introducing a triple internal standard method, which instead of additional ladder separations needs co-injection of labeled maltooligosaccharide standards and uses computational models fitted to these standards added to the sample to assign GU values to the migration times of analytes in the electropherogram (Jarvas *et al.*, 2018). By this, one can significantly reduce sample processing time, power- and reagent consumption, rendering CE sustainable. This CE-LIF based oligosaccharide analysis technique has become so efficient, that it is possible not only to

separate glycans of different sizes, but also alpha and beta linked or positional isomers of N-glycans comprised of the same monosaccharides (Donczo *et al.*, 2016 and Donczo *et al.*, 2019).

Recent emergence in commercialization of miniaturized digital technology (Foster *et al.*, 2019) and 3D printing (Kalsoom *et al.*, 2018) allows analytical laboratories to come up with custom solutions to improve their performance (Fichou and Morlock, 2018) and at the same time go green. Most commercial capillary electrophoresis devices are using either photodiodes or PMTs as optical detectors or require large additional parts in case of laser induced fluorescent detection. Advancements in LED technology, microcontroller platforms and CMOS or CCD-based imaging detectors offer similar efficiency to traditional techniques, but at lower price, smaller footprint, and efficient data post processing capability (Szarka *et al.*, 2019), where the signal can be reprocessed if necessary for optimized data evaluation (i.e., saturated sample signal can be reprocessed without repeating the measurements). This provides an opportunity to utilize novel hardware and software solutions for solving analytical challenges in a sustainable manner. In this paper we intend to further emphasize the sustainability of capillary electrophoresis with LED-induced fluorescence detection. For this, we compare the same PNGase F released, APTS-labeled immunoglobulin G N-glycan separation results obtained with System A, a home built low consumption CE device, operated semi automatically (Szarka and Guttman, 2017) and System B, a fully automated, high end commercial device. Our analysis shows that with some limitations both techniques show potential in the separation milieu.

2. Methods

2.1. Chemicals

Glacial acetic acid, sodium-cyanoborohydride (1 M in THF), HPLC grade water and acetonitrile were all purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). PNGase F enzyme was purchased from Asparia Glycomics (San Sebastian, Spain). Human Immunoglobulin G1 was obtained from Molecular Innovations (Novi, MI, U.S.A.). Fast Glycan Labeling and Analysis Kit was purchased from Sciex (Brea, CA, U.S.A.), with the following reagents included in it: 8-aminopyrene-1,3,6-trisulfonic acid (APTS) tagging dye, the maltooligosaccharide ladder, maltose, and magnetic beads.

2.2. Sample Preparation

First, 1.0 mg of human Immunoglobulin G1 (hIgG1) were PNGase F digested, then the cleaved N-glycans were APTS labeled, according to the procedure published by Szigeti *et al.* (2017).

2.3. Capillary Electrophoresis

- System A: Separations were performed with a previously described custom-made capillary electrophoresis unit, equipped with LED-induced fluorescence imaging detection system (Szarka and Guttman, 2017). Effective capillary length was 20 cm (total length 30 cm), 50 μ m ID Bare Fused Silica capillary. All separations were performed at 25 °C. Separation voltage was 12 kV in reversed polarity mode (cathode at the injection side). Two-stage manual electrokinetic sample injection method was applied to utilize stacking effect: (i) 1 kV for 1 s water injection, (ii) sample injection at 3 kV for 3 s. The N-CHO separation gel buffer (SCIEX) served as background electrolyte. A custom written software package was used for data acquisition and processing, following our method described earlier (Szarka and Guttman, 2017).
- System B: Reference separations were performed on a PA800 Plus Pharmaceutical Analysis System (SCIEX). Effective capillary length was 20 cm (total length 30 cm), 50 μ m ID Bare Fused Silica capillary as a preassembled cartridge from the kit. Cartridge temperature was set to 25 °C for all runs. Other injection and separation parameters were the same as for system A. The background electrolyte was the N-CHO separation gel buffer (SCIEX) and the 32Karat software package (version 10.1, SCIEX) was used for data acquisition and processing.

3. Results and discussion

As our environment is continuously changing, there is an increasing demand for innovative solutions in all branches of science. These solutions should be more robust, more efficient, or more sensitive from the methodological standpoint, but are also called to reduce resource exploitation of Earth, to insure sustainable future. New sample processing methods can lower the required amount of potentially pollutant chemical components, while signal processing optimization can help reducing either analysis time or even the number of necessary separation runs and, by this, the amount of chemicals used.

Results reported in this paper support both tasks by applying improved sample preparation techniques and utilizing a custom-made capillary electrophoresis unit with LED induced fluorescent imaging detection. Separation results were compared to the gold industry standard of PA800 Plus Pharmaceutical Analysis system (See Figure 1).

Although the two systems are considered to be far from each other in terms of versatility and quality (main instrument characteristics are summarized in Table 1); results clearly show meaningful electropherograms generated by either instrument.

Table 1. Capillary electrophoresis sustainability characteristics comparison of a commercial and a custom-built instrument

Instrument type	PA800 Plus (Reference Instrument)	Custom made CE-LedIF
Size, weight	74x64x70 cm, over 100 kg	40x250x25 cm, ~10 kg
Power requirements	over 1 kW	below 100 W
Separation conditions	safe, precise, temperature controlled	less precise, no temperature control
Automation level	programmable autosampler	semi-automated, no autosampler
Signal Processing	nonadjustable dynamic range / runs	adjustable dynamic range/ runs
Overall quality	High end, industrial instrument	Low-end home- made system

Both instruments separate analytes prepared with identical sample preparation procedures. Both systems utilize excitation wavelength with a maximum at 488 nm (LED in case of the custom-built system, Laser for the PA800 Plus instrument) for the excitation of the APTS labeled N-glycans and bandpass filters with an emission maximum at 520 nm in both cases.

The custom-built CE (Figure 1, trace A) utilized fluorescent time-lapse imaging through its CCD detector employing image analysis - pixel intensity calculation in 8-bit - as the basis of Relative Fluorescence Unit (RFU) values. The PA800 Plus instrument was equipped with a PMT detector module using photon counting as the basis of the expressed RFU values (Figure 1, trace B).

Both instruments were able to separate and detect sufficiently the following N-glycan molecules, also marked in Figure 1: 1 & 1' = FA2G2S2, 2 & 2' = FA2BG2S2, 3 & 3' = A2G2S1, 4 & 4' = FA2G2S1, 5 & 5' = FA2BG2S1, 6 & 6' = FA2, 7 & 7' = FA2B, 8 & 8' = FA2[6]G1, 9 & 9' = FA2[3]G1, 10 & 10' = FA2B(6)G1, 11 & 11' = FA2B[3]G1, 12 & 12' = FA2G2, 13 & 13' = FA2BG2. Although the reference separation (Figure 1, trace B) shows much smoother signal with sharp symmetrical peaks, the much cheaper and less precisely controlled custom built setup (Figure 1, Trace A) was capable of producing qualitative results with much smaller power requirements, at a significantly smaller footprint, without temperature control, but at the price of losing standard quality assurance and key automation steps (see Table 1).

Figure 1. Comparative separation of released and APTS labeled hIgG1 N-glycans; x-axis represent migration times; y-axis values are in Relative Fluorescent Unit (RFU) values. Upper trace: Home-built CE-LedIF unit (System A). Lower trace: PA800 Plus Pharmaceutical Analysis System with Laser induced Fluorescence Detection (System B). In both cases separations were performed at 12 kV in 20 cm effective length (30 cm total length), 50 μ m ID BFS capillary. N-CHO separation gel buffer was used as background electrolyte.

5. Conclusions

Advancement of technology over recent years made it possible to utilize consumer products, such as smartphones, 3D printers, etc. in the field of liquid separation science to produce highly customized devices for analytical chemistry applications. This advancement allows to compare chromatography results obtained by custom built devices relative to commercial instruments, but has become a driving force to use acquired knowledge to do science in a different way, in a preferably sustainable manner. In this paper a previously described, custom made, 3D printed, modular, semi-automated imaging based CE-LedIF instrument was compared to an industry gold standard, fully automated high end machine by analyzing PNGase F digested, APTS labeled human Immunoglobulin G type 1 N-glycans via one of the most advanced and sustainable sample preparation methods. As a matter of fact, the purpose of this study was to prove that with the availability of credit card sized minicomputers and highly sensitive, inexpensive CCD cameras it is possible to accomplish sufficient magnitude of signals and convenient resolution to enable data analysis with limitations and sustainability in capillary electrophoresis equipped with LED induced fluorescent detection.

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