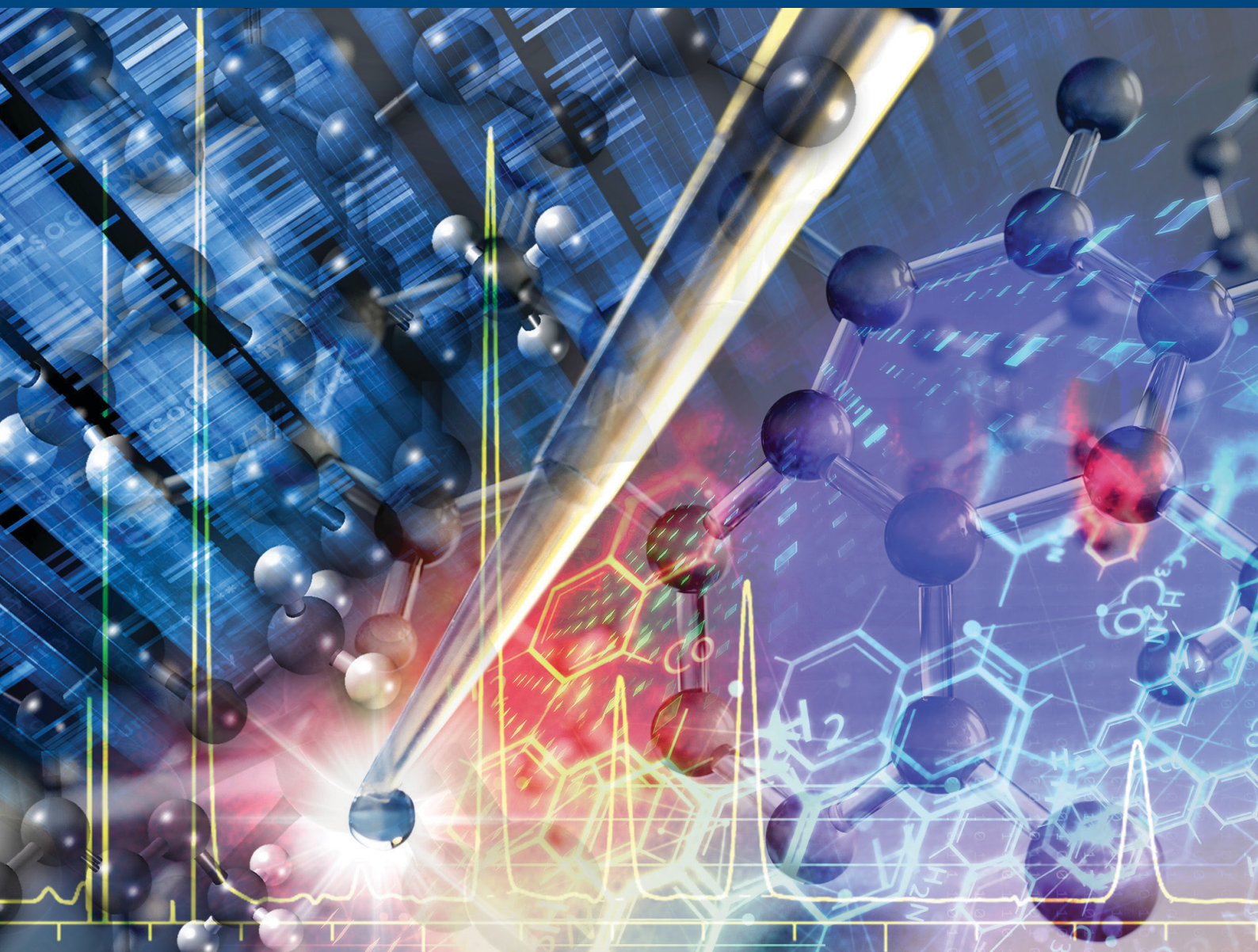


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RESEARCH ARTICLE

In capillary labeling and online electrophoretic separation of N-glycans from glycoproteins

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In this study, we present a new approach for in-capillary fluorescent labeling of N-glycans prior to their analysis with CE coupled with laser-induced fluorescent detection. This integrated approach allows using a CE capillary as a microreactor to perform several steps required for labeling glycans with 8-aminopyrene-1,3,6 trisulfonic acid and at the same time as a separation channel for CE of fluorescently labeled glycans. This could be achieved through careful optimization of all different steps, including sequential injections of fluorescent dye and glycan plugs, mixing by transverse diffusion of laminar flow profiles, incubation in a thermostatic zone, and finally separation and detection with CE. Such a complex sample treatment protocol for glycan labeling that is feasible thus far only in batchwise mode can now be converted into an automated and integrated protocol. Our approach was applied successfully to analyze fluorescently labeled N-linked oligosaccharides released from human immunoglobulin G and rituximab, a monoclonal antibody used for cancer treatment. We demonstrated the superiority of this in-capillary approach over the conventional in-tube protocol, with fourfold less reagent consumption and full automation without remarkable degradation of the glycan separation profile obtained by capillary electrophoresis.

KEYWORDS

8-Aminopyrene-1,3,6 trisulfonic-acid, Capillary electrophoresis, Glycosylation, In-capillary labeling, N-glycans

1 | INTRODUCTION

The regular functioning and activity of glycoproteins, which represent half of all secretory and human cel-

lular proteins, can be impaired by altered glycosylation [1, 2]. Indeed, a variation in the glycoform proportion of a given glycoprotein may lead to biological consequences, alteration/degradation of therapeutic effects (for instance, monoclonal antibodies used for disease treatment) or be the result of a pathological state [3]. Analysis of glycans released from glycoproteins, therefore, plays an important role in the quality control of therapeutic glycoproteins and diagnostic purposes [4, 5]. Until now, high-performance liquid chromatography with fluorescence

Abbreviations: 2-PB, 2-picoline-borane; APTS, 8-aminopyrene-1,3,6 trisulfonic-acid; FLR, fluorescence detection; G12, maltododecaose; G6, maltohexaose; G9, maltononaose; HAc, acetic acid; IgG, human Immunoglobulin G; IS, ionic strength; LPE, liquid-phase extraction; mAbs, monoclonal antibodies; MD ladder, malto-oligosaccharides; TDLFP, transverse diffusion of laminar flow profiles.

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detection (HPLC-FLR) [6], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [7, 8], and liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) [9, 10] have been frequently used methods for this purpose. Recently, with the introduction of commercial kits [11, 12], capillary electrophoresis (CE) coupled with laser-induced fluorescent (LIF) detection has become a well-recognized method for glycan analysis [13–15]. In a standard CE-LIF protocol, released glycans are normally labeled with a negatively charged fluorophore (8-aminopyrene-1,3,6-trisulfonic-acid, APTS, in most cases [16]) via a reductive amination reaction, followed by washing of APTS-labeled glycans and removal of residual fluorophores prior to their offline separation by CE-LIF [17]. The batchwise in-vial method for glycan sample treatment, employing magnetic microbeads as the solid support to carry out these steps, has now become the reference protocol [18–20], being exploited in commercial kits [11]. Additionally, based on a similar batchwise method, Perkin-Elmer developed a microchip-CE platform to release and label N-glycans prior to their separation using microchip electrophoresis with a 96-well microtiter-plate-based setup [21]. While offline batchwise protocols for glycoprotein digestion and glycan labeling have been well established, there are nevertheless some considerations when working with these approaches, notably laborious in-tube operations, mismatch of the working volumes between the sample treatment and separation steps, possible cross-contamination or analyte loss when transferring the analytes from one step to another. Integration of all sample pretreatment and separation steps into a microchannel is therefore of high interest to overcome the aforementioned limitations.

In a related context, in-capillary sample treatment has gained particular attention as an alternative to conventional batchwise sample processing for downstream CE analysis [22–25]. With this approach, the capillary used for CE separation serves at the same time as a microreactor to carry out different sample treatment steps upstream. Hydrodynamic and/or electroosmotic flows, upon application of pressurization and/or high voltage, respectively, are used in this case as driving forces to introduce samples and reagents and trigger mixing inside the capillary. Two main strategies were accordingly developed for this purpose, including transverse diffusion of laminar flow profiles (TDLFP), where a hydrodynamic flow is created to deform the boundaries of reagent and analyte plugs to facilitate diffusion and thus mixing [26, 27], and electrophoretically mediated microanalysis (EMMA), in which compounds are mixed via the difference in their electrophoretic mobilities under an electric field [28–30]. The application of EMMA is limited when some reactants are sensitive to the electric field when the electrophoretic

mobilities of several reactants are similar or the reaction requires too many reaction components. Among these two strategies, TDLFP is often the method of choice with better performance for in-capillary mixing because it has no constraints regarding the electrophoretic mobility of the involved reactants [31, 32]. Longitudinal diffusion in the capillary with TDLFP is much faster and more pronounced than lateral diffusion and is less influenced by molecular sizes, thus rendering the mixing very efficient [33]. This interesting approach nevertheless has never been explored for a complex labeling reaction requiring several steps and reactants for glycans, to the best of our knowledge. Based on this rationale, we present in this study a new in-capillary strategy for the fluorescent labeling of N-glycans by APTS, followed by their subsequent CE-LIF analysis. Such a reaction in the capillary format is very challenging, as it involves the mixing of several components under extreme conditions (i.e., elevated temperature and prolonged incubation) with the risk of gas formation that can damage the mixing and subsequent CE analysis. These are not trivial, as a multicomponent reaction performed with TDLFP requires careful optimization of each component concentration and position, depending on their diffusion, to avoid mixing too early or too late. Extreme attention is also required to ensure that all these reactants and generated byproducts do not interfere with the expected peaks of target analytes (i.e., glycan peaks in our case) during the subsequent CE analysis. The novelty of our work is therefore the adaptation of this particular multicomponent glycan and quite slow labeling reaction to the TDLFP mode of mixing and subsequent online CE separation of the labeled glycans. Our approach was applied to the N-glycan mapping of human immunoglobulin G (IgG) and rituximab, a monoclonal antibody used for cancer treatment.

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents, and samples

Reagents for the preparation of glycans (malto-oligosaccharides (MD ladder), fluorescent dye 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS), and magnetic beads) were obtained from the Fast Glycan Analysis and Labeling kit of Sciex (Villebon sur Yvette, France). PNGase F (namely, peptide N-glycosidase F), 1 M sodium cyanoborohydride (NaBH_3CN) in tetrahydrofuran, 2-picoline-borane (2-PB), acetic acid (HAc), acetonitrile and human immunoglobulin G were obtained from Sigma-Aldrich (Lyon, France). Maltohexaose (G6, > 95% m/m) was purchased from Biosynth Carbosynth (Berkshire, United Kingdom).

Maltonaose (G9, > 98% m/m HPLC) and maltodecaose (G12, > 95% m/m HPLC) were purchased from Elicityl (Crolles, France). All chemicals for the preparation of buffers were of analytical or reagent grade and purchased from Sigma–Aldrich (Lyon, France). For the preparation of background electrolyte (BGE) solutions, triethanolamine and citric acid were also purchased from Sigma. Rituximab was purchased from Roche (Basel, Switzerland). All aqueous solutions were prepared with deionized water, which was purified through a Direct-Q3 UV purification system (Millipore, Milford, MA, USA). All prepared buffers were filtered through a 0.22 μm nylon membrane (VWR) before use.

2.2 | Apparatus and material

The CE experiments for glycan labeling and separation were performed with a P/ACE MDQ instrument (SCIEX, Framingham, MA, USA) equipped with a ZETALIF detector (λ ex: 488 nm, λ em: 520 nm) from Adelis (Toulouse, France). A 32 Karat version 8.0 software (Sciex) was used to carry out the instrument control and data acquisition. Fused silica capillaries of 50 μm od and 375 μm id from Polymicro (CM Scientific, Silsden, UK) were used for in-capillary glycan labeling and CE-LIF separation. The monitoring of the labeling reaction was implemented under a Macro Objective (MLH-10X) mounted on a CMOS camera (aca1300-60 gm, Basler) and white LED back-light illumination (Schott Lighting and Imaging) using UV-transparent fused silica capillaries of 365 μm od and 50 μm id (Polymicro technologies Phoenix AZ, United States). Viscosity measurements were conducted on a P/ACE MDQ Instrument (Sciex) coupled to a UV detection set at 200 nm. A freeze dryer (Heto Power Dry LL 1500, Thermo Electron Corporation, Warwickshire, U.K.) was used for lyophilization of malto-oligosaccharide samples. Buffer ionic strength (IS) calculations were based on simulations with the computer program PhoeBus (Analis, Suarlée, Belgium).

2.3 | Methods

2.3.1 | In-tube fluorescent labeling of malto-oligosaccharides

APTS-labeled malto-oligosaccharides (G6, G9 and G12) were prepared to estimate in-capillary labeling yields. The offline labeling protocol was adapted from those reported by Guttman's group. [34, 35]. Briefly, 5 nmol of (lyophilized) malto-oligosaccharide was added to a mixture of 2 μL of 0.2 M APTS in 20% HAc (v/v) and 2 μL of 1 M

NaBH₃CN in tetrahydrofuran and incubated for 50 min at 45°C. G12 (5 nmol), used as an internal standard (APTS-G12), was also labeled with APTS separately (18 h at 37°C) using a protocol similar to that reported in [34]. After incubation, the mixtures were diluted 100-fold with deionized water and stored at –20°C until subsequent dilution and analyses.

2.3.2 | In-tube enzymatic release of glycoprotein-derived glycans

Ten microliters of glycoprotein (IgG or rituximab, 10 mg/mL in water) solution was mixed with 7 μL of the denaturing solution prepared according to the protocol from the Sciex kit. The mixture was then incubated for 10 min at 70°C. Then, 17 μL of the digestion solution containing PNGase F at a final activity of 1.5 U, prepared according to Sciex's protocol, was added, followed by a second incubation for 60 minutes at 50°C. The obtained sample containing released N-glycans was then mixed with magnetic clean-up beads from the kit (after removal of supernatant from 50 μL of bead suspension) and vortexed for 10 s at the maximum speed. Then, 200 μL of acetonitrile was added to the sample tube and vortexed for 10 s to jam the released N-glycans onto magnetic beads. The supernatant was then removed while the magnetic beads containing N-glycans on their surface were retained using a magnetic stand. N-glycans captured on magnetic beads were then eluted with 16 μL of water, and 4 μL of pure HAc (concentration of 17.5 M) was subsequently added prior to the in-capillary labeling step.

2.3.3 | CE-LIF of fluorescently labeled oligosaccharides and glycans

Analyses of APTS-labeled glycans or malto-oligosaccharides were carried out with our recently developed BGE composed of triethanolamine/citric acid (IS of 100 mM, pH 4.75) [36]. These separations were implemented using a fused-silica capillary of 50 μm id, with a total length (L_{tot}) of 60 cm and an effective length (L_{eff}) of 50 cm, under a separation voltage of –25 kV. The fused silica capillary was preconditioned by rinsing with water for 5 min, 1 M NaOH for 5 min, water for 5 min, 1 M HCl for 5 min, water for 5 min, and the BGE for 30 min prior to the first use. Between the two runs, the capillary was rinsed with the BGE for 5 min under pressure, and then a voltage of –25 kV with a pressure of 6.9 kPa was applied for 8 min followed by rinses (at 172.4 kPa under the cartridge temperature of 45°C) with water for 5 min, 0.1 M NaOH for 5 min, water for 5 min, and BGE for 5 min.

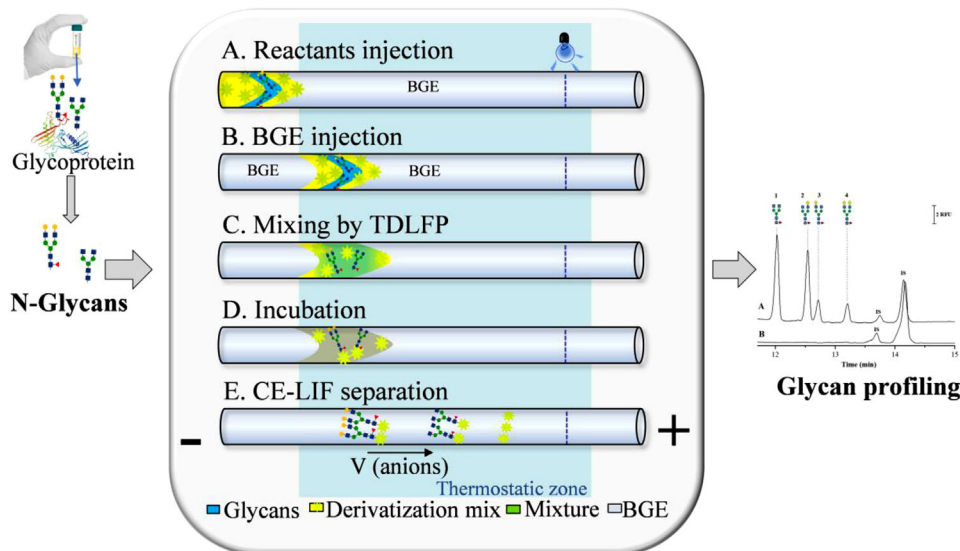


FIGURE 1 Schematic drawing of the protocol for the in-capillary APTS labeling of glycans

2.3.4 | In-capillary fluorescent labeling and CE-LIF separation of malto-oligosaccharides and glycans

The in-capillary labeling method proposed here was adapted from the mixing procedure based on transverse diffusion of laminar flow profiles (TDLFP) [33]. This was done according to the sandwich-injection strategy proposed by Krylov et al. [37], allowing subsequent injections of APTS derivatization solution, glycans, APTS solution and a long plug of BGE into the capillary preheated to 45°C to improve the subsequent TDLFP mixing. The optimized protocol consisted of injection of APTS solution (with an optimized APTS/glycan molar ratio of 29150 and 12.5 mM NaBH₃CN in 20% HAC (v/v)) under 2.8 kPa for 3.5 s, sample solution (i.e., malto-oligosaccharides or protein-released glycans in 20% v/v HAC) under 5.5 kPa for 3.5 s, APTS solution under 2.8 kPa for 3.5 s, and BGE under 5.5 kPa for 90 s. After each injection, the capillary inlet was dipped in a water vial to avoid cross-contamination. TDLFP was triggered by applying a pressure of 5.5 kPa for 3.5 s to render the injected plugs substantially parabolic. Each injected plug then penetrates the zone next to it, forming longitudinal interfaces between two adjacent plugs. The capillary was then incubated for 50 min at a temperature of 45°C. After the labeling reaction, the cartridge was cooled to 25°C (over 5 min) before triggering the CE-LIF separation of in-capillary labeled glycans under a separation voltage of -25 kV. Details on the measurement of sample plug volume and solution viscosities can be found in the ESI. The labeling yields obtained with in-capillary labeling and online CE-LIF of labeled malto-oligosaccharides were compared to those from in-tube labeling and offline

CE-LIF of these compounds using the following equation:

$$Y = (S_{\text{online}} \times C_{\text{offline}}) / (S_{\text{offline}} \times C_{\text{online}}) \times 100\% \quad (1)$$

where Y is the ratio between the labeling yields achieved with the in-capillary approach (for G6, G9 or G12) and that obtained with the in-tube protocol. The S_{online} is for the peak area of the target malto-oligosaccharide obtained with on-line labeling, the C_{online} is for the malto-oligosaccharide concentration used for on-line labeling, the S_{offline} is the peak area of the target malto-oligosaccharide obtained with offline labeling, and the C_{offline} is for the malto-oligosaccharide concentration used for offline labeling.

3 | RESULTS AND DISCUSSION

3.1 | Protocol development for in-capillary labeling of malto-oligosaccharides/glycans

To perform the in-capillary labeling of N-glycans prior to their electrophoretic separation by CE-LIF, we employed the TDLFP strategy, which uses pressure to introduce reactants and analyte solutions into the capillary through successive plugs [37]. The reactants are mixed mainly by transverse diffusion after these successive injections. The protocol for in-capillary fluorescent labeling of glycans prior to their separation with CE-LIF is illustrated in Figure 1. It starts with sequential injections of one APTS reaction mixture containing the dye, the reducing agent (NaBH₃CN, required for amine reduction of APTS during

the labeling reaction) and the acidic catalyst acetic acid (HAc), then the glycan solution to be labeled and again the APTS reaction mixture. This is followed by mixing via transverse diffusion and incubation under the desired temperature set on the capillary cartridge. Several challenges had to be considered and overcome when realizing such an in-capillary protocol. First, efficient mixing of different reactants inside the capillary was not trivial due to the presence of boundaries between two different plugs, which is not the case with in-tube mixing. The longer the plugs of APTS and/or glycan solution are, the more difficult it is to obtain homogeneous mixing. While boundaries between plugs had to be as long as possible to favor diffusion and efficient mixing, this provokes intense parabolic flow deformation, which could be detrimental to the separation resolution during the subsequent CE separation step. An optimized condition therefore had to be found to allow maintaining a high labeling yield and, at the same time, good stacking of the analyte zone (for satisfying separation performance). Accordingly, the conductivity of the sample matrix, which is strongly influenced by the presence of APTS (bearing a triple negative charge) and HAc, should be kept as low as possible or, at least, much lower than that of the BGE. Second, we noticed that the presence of the reducing agent NaBH_3CN in the capillary under an elevated temperature provoked the formation of gas bubbles (see the reaction in Figure S1), leading to a current drop during the subsequent CE analysis of APTS-labeled glycans. This was indeed the case when using a high concentration of NaBH_3CN (final concentration of 167 mM after mixing, normally employed for in-tube glycan labeling) (Figure S1). A significant reduction in the NaBH_3CN concentration by 20-fold down to 8 mM could solve this problem. An effort was also made to replace NaBH_3CN with another reduction agent, 2-picoline-borane (2-PB), which was also reported to be nontoxic [17]. The labeling performance obtained for 2-PB, calculated from the peak intensities from the CE-LIF electropherograms, was, however, not as high as that with NaBH_3CN (twofold lower, Figure S2), and more bubble formation was observed with 2-PB. Our preliminary tests with NaBH_3CN at the optimized concentration of 8 mM demonstrated that in-capillary labeling of malto-oligosaccharides (G6, G9, and G12) using such a low NaBH_3CN concentration and their subsequent CE-LIF separation are feasible (Figure S3). G6 to G12 were chosen as good standards to mimic N-glycans, as their electrophoretic migration behavior (once labeled) corresponds to that found for the most common N-Glycan structures. While the preliminary results were encouraging, further optimization of in-capillary mixing and reagent concentrations were required to obtain improved and satisfactory glycan labeling yields. In a related concern, the presence of APTS at

high concentrations and in excess, which is mandatory for the APTS labeling reaction, leads to signal saturation and unwanted overlapping of target glycan peaks in the subsequent CE-LIF detection step. We therefore implemented careful optimizations of different parameters, including molar ratios of APTS/glycan in the mixture, volume ratios of APTS/sample/APTS plugs, number of injected plugs (from 3 to 11), and in-capillary incubation time and temperature, to overcome all aforementioned hurdles. The labeling efficiencies under various conditions, which were calculated from the peak areas of G6, G9 and G12 obtained with in-capillary APTS labeling and online CE-LIF of these compounds, were compared with those obtained via in-tube labeling and offline CE-LIF separation (Figure 2). It was found that an increase in the temperature of the capillary cartridge to 45°C allowed an improvement in the mean labeling yield (Figure 2A), as the standard deviation of yields were all less than 5% at 45°C. This is also consistent with a trend reported in the literature of increasing the proportion of labeled glycans with increasing temperatures [38]. Higher temperatures (up to 50°C as normally used for in-tube labeling [18]) were also tested but were found to result in a more pronounced formation of gas bubbles (induced by reaction with NaBH_3CN) and current leakage that hinders the subsequent online CE-LIF step. The molar ratios between APTS and glycans in the plugs injected into the capillary were then optimized to seek a compromise between resolution between two adjacent malto-oligosaccharide peaks and labeling efficiency (Figure 2B). A low APTS/glycan ratio of 25,000 led to poor labeling yield (yield < 25%), whereas a molar ratio (145,800) that was too high resulted in decreased peak resolution between G6 and G9 due to the residual and byproducts of APTS that degrade the CE-LIF separation of labeled malto-oligosaccharides. The incubation time is also an important factor to consider. Labeling yields increased from 20 to 50 min, but a prolonged duration of 50 min allowed an equivalent labeling performance compared to that obtained with the in-tube protocol using the same incubation time (i.e., relative labeling yield of 97% compared to that of the in-tube labeling protocol, Figure 2C). As a result, the best in-capillary labeling of malto-oligosaccharides was achieved using 8 mM NaBH_3CN , APTS/glycan molar ratio of 58,300, and incubation at 45°C over 50 min.

Diffusion of reagents between plugs sequentially injected in the capillary is another factor that influences the mixing performance [39, 40]. Different volume ratios between APTS mixture and glycan sample plugs, as well as the number of plugs (3, 7 and 11 plugs) injected, were therefore tested (see Figure S4). The best mixing, reflected by the highest labeling efficiency, was achieved with the plug length ratios for APTS:sample:APTS of 1:2:1 (see

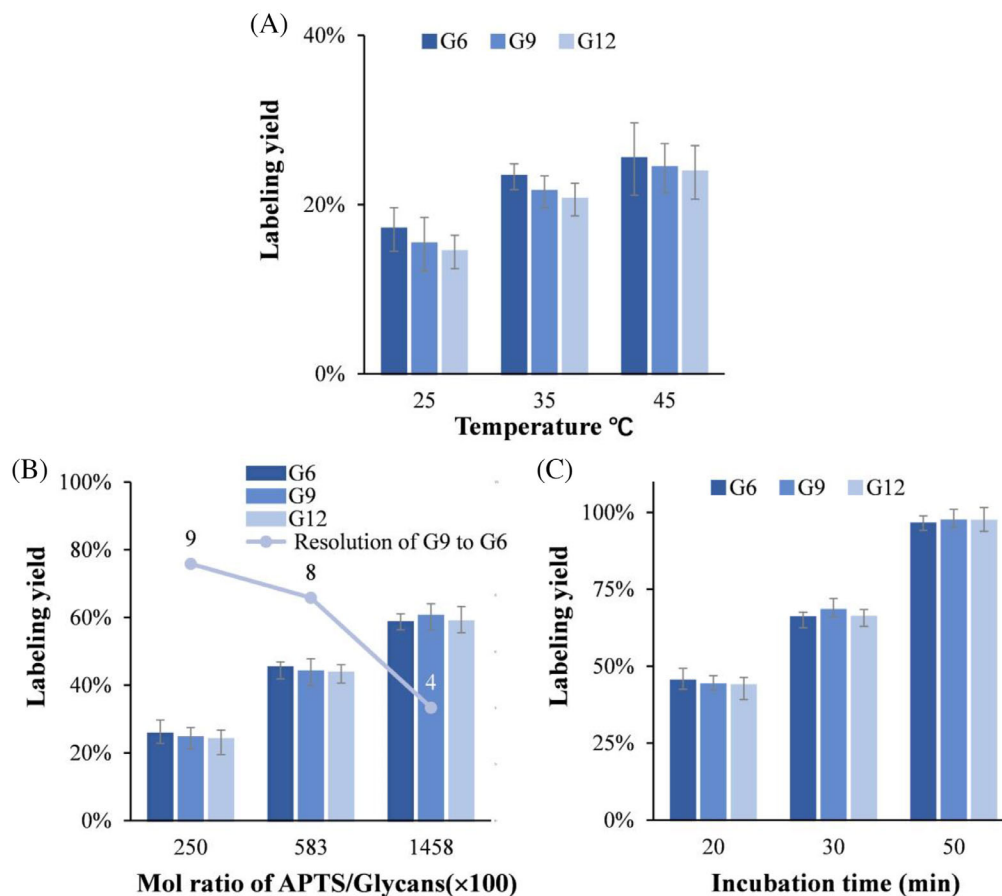


FIGURE 2 Effects of reaction conditions on labeling yields of APTS-labeled G6, G9, and G12 mixtures. Effect of (A) temperature. (B) Molar ratio of APTS/glycans. (C) Incubation time. CE-LIF conditions: (A) 8 mM NaBH₃CN in 20% HAc (v/v), incubation time 30 min, mol ratio of APTS/Glycans = 25,000 (glycan concentration 6 μM), plugs volume ratio APTS:Glycans:APTS = 1:1:1. (B) 8 mM NaBH₃CN in 20% HAc (v/v) at 45°C, incubation time 30 min, glycan concentration 6 μM, plug volume ratio APTS:glycan:APTS = 1:1:1. (C) 8 mM NaBH₃CN in 20% HAc (v/v) at 45°C, mol ratio of APTS/Glycans = 58,300 (glycan concentration = 6 μM), plugs volume ratio APTS: Glycans: APTS = 1:1:1

tables S1 and S2 and the method for calculation volume ratios of injected plugs), regardless of the number of plugs injected. It is seen that a minimum injection number of 3 plugs already offered sufficient mixing efficiency. These injection conditions with a plug length ratio of 1:2:1 and a plug number of 3 (namely, APTS-glycans-APTS) were therefore chosen for further investigation of labeling performance. Figure 3 shows the comparison of peaks of different malto-oligosaccharides obtained with the in-capillary approach under our optimized conditions and those from the in-tube protocol under equivalent incubation time (50 min) and temperature (45°C). Similar performance in terms of peak height and separation resolution (G9 to G6 peaks) was achieved in both cases. The superiority of this in-capillary approach was demonstrated with fourfold less reagent consumption and full automation without degradation of the glycan separation profile obtained by CE-LIF compared to the conventional in-tube batchwise method. The relative peak area of each

TABLE 1 In-capillary and in-tube labeling of G6, G9, and G12 peak area ratios

| Peak area ratio ^a | In-capillary | In-tube |
|------------------------------|--------------|---------|
| AG6 | 35% | 36% |
| AG9 | 33% | 33% |
| AG12 | 32% | 31% |

^aEstimated by the ratio of the peak area (A) of one given Gi (AGi) to the sum of peak areas (AG6+AG9+AG12).

individual G6, G9 or G12 signal (i.e., the ratio between the peak area of one given malto-oligosaccharide to the sum of the peak areas of G6, G9 and G12) was found to be similar for both methods (Table 1). This demonstrates that our method did not lead to unwanted labeling bias for any particular malto-oligosaccharide and that the labeling operates with similar efficiency regardless of the oligosaccharide size.

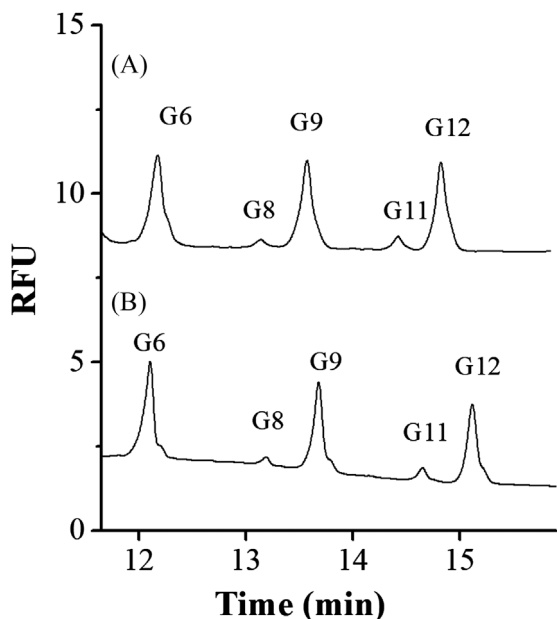


FIGURE 3 (a) Electropherograms of in-capillary labeling of G6, G9, G12 mixture under the optimal conditions: 8 mM NaBH₃CN in 20% HAc (v/v) at 45°C, incubation time 50 min, mol ratio of APTS/Glycans = 29,150 (glycan concentration: 6 μM), plugs volume ratio APTS: Glycans: APTS = 1:2:1. (b) In-tube labeling of the G6, G9, and G12 mixtures at 45°C for 50 min (glycan concentration: 5.5 μM)

3.2 | Determination of N-glycans released from glycoprotein

Our in-capillary approach was then applied for the analysis of N-glycans released from rituximab, a monoclonal antibody used to treat certain autoimmune and different cancer diseases. In monoclonal antibodies (mAbs) in general and rituximab in particular, glycosylation represents only 2-5% of the total mass of the protein and can have a significant influence on the effector functions of mAbs [41]. The rituximab glycosylation profile is therefore considered a critical quality attribute of mAbs. Figure 4 shows the glycan profile of rituximab obtained with our in-capillary approach. Three main peaks attributed to rituximab glycans were identified as “G0F(H3N4F1), G1F(H4N4F1)” (i.e., two structural isomers), and G2F(H5N4F1) according to the literature. This glycan profile is indeed similar to that obtained with in-tube sample processing and offline CE-LIF analysis [42]. These major rituximab glycans are expected to be the most abundant, each accounting for more than 10% of the total glycan content. The percentage of each major glycan, calculated from the results obtained with in-capillary labeling of the rituximab glycans (Table 2), matches well with that reported previously by CE-ESI-MS analysis (e.g., 42.7% for G0F(H3N4F1), 46.5% for G1F(H4N4F1), and 10.8% for G2F(H5N4F1) [41]. Note that the difference in peak shape for G12 in

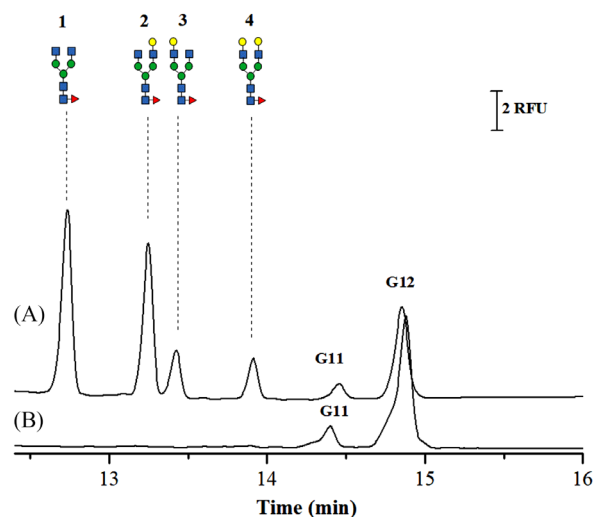


FIGURE 4 In capillary labeling of rituximab-released N-glycans (from a 10 μg/μL protein sample diluted twofold after N-glycan release) (a) and blank without rituximab (b). An internal standard APTS - G12 was added to the released glycan solution and blank before injection. At a final concentration of 200 mM, the G12 sample also contained traces of maltopentaose APTS - G11. CE conditions: 8 mM NaBH₃CN in 20% HAc (v/v) at 45°C, incubation time 50 min, mol ratio of APTS/Glycans = 29,150 (glycan concentration: 6 μM), plugs volume ratio APTS: Glycans: APTS = 1:2:1. Symbols: ■, N-acetylglucosamine; ●, mannose; ●, galactose; ◆, N-acetylneuraminic acid; ▲, fucose

Figure 4a,b could be due to the different sample matrices, which may lead to different stacking performances and thus slight modification of peak shapes. This difference is more evident for the long-migrating species peaks where electromigration dispersion and diffusion are more pronounced, which is the case for G12. Our approach was also applied for labeling and online analysis of N-glycans from human IgG (Figure 5). These IgG glycans are known to be mainly biantennary complex glycans with low terminal sialylation (5-10%) and high core fucosylation (> 92%), with a small proportion containing a bisecting GlcNAc (11%) [43]. The CE-LIF profile of IgG glycans obtained with our approach is very similar to those already reported that used conventional tube-based protocols and CE profiling [36, 44]. From the electropherograms obtained for human IgG, the relative proportion of the five most abundant glycans was calculated and compared to those obtained with in-tube labeling from the literature (Table 2). Similar results were found for all these IgG glycans with the in-capillary or in-tube method. The matching results between the two approaches for both tested glycoproteins indicate that the automated in-capillary glycan labeling method is equally performant to the in-tube and offline method, which is of extreme importance for accurate glycan profiling and analysis.

TABLE 2 Peak area ratios of glycans among the most abundant ones of rituximab and human IgG, calculated from electropherograms obtained from the in-capillary approach. These values are compared to those obtained with in-tube labeling and CE-ESI-MS analysis for rituximab [41] and CE-LIF analysis for IgG [44]

| NO. | Glycan structure ^b | In capillary labeling | | In-tube labeling | |
|--------------------------|-------------------------------|-----------------------------|---------------|-----------------------------|---------------|
| | | Area ratio (%) ^c | SD (n = 3, %) | Area ratio % ^{c,d} | SD (n = 3, %) |
| Rituximab | | | | | |
| 1 | G0F(H3N4F1) | 44.2 | 0.7 | 42.7 | 1.8 |
| 2 | GIF(H4N4F1) | 35.4 | 0.4 | n.r. | n.r. |
| 3 | GIF(H4N4F1) | 10.7 | 0.5 | n.r. | n.r. |
| Sum ^a of 2, 3 | – | 46.2 | 0.6 | 46.5 | 1.1 |
| 4 | G2F(H5N4F1) | 9.6 | 0.1 | 10.8 | 0.9 |
| IgG | | | | | |
| NO. | Glycan structure ^b | Area ratio (%) ^c | SD (n = 3, %) | Area ratio % ^{c,e} | SD (n = 3, %) |
| 1 | 11.3 | 0.8 | 8.8 | 0.6 | |
| 2 | 22.3 | 1.9 | 27.1 | 0.6 | |
| 3 | 26.5 | 0.4 | 27.6 | 0.4 | |
| 4 | 17.9 | 1.7 | 15.0 | 0.5 | |
| 5 | 22.0 | 1.1 | 21.5 | 0.3 | |

^aIn this work, the two glycans corresponding to peaks 2 and 3, which are structural isomers, are not considered separately.

^bSymbols: , N-acetylglucosamine; , mannose; , galactose; , N-acetylneuraminic acid; , fucose.

^cEstimated by the ratio of peak area (A_i) of one released glycan (i) to the sum of the most abundant released glycan peak area (i.e., $(A_1+A_2+A_3+A_4)$ for rituximab and $(A_1+A_2+A_3+A_4+A_5)$ for IgG glycans).

^dCalculated from ref. [41].

^eCalculated from ref. [44].

SD: standard deviation.

n.r., not reported.

4 | CONCLUDING REMARKS

Successful in-capillary APTS labeling of glycans was developed and was found to be compatible with online CE-LIF analysis of fluorescent N-glycans. We demonstrated that the TDLFP-based approach offered a labeling efficiency similar to that obtained with the in-solution reaction and that the in-capillary process did not induce any bias when labeling glycans of different sizes or structures. The application of the method to IgG and rituximab demonstrated similar glycan profiles as with the traditional offline approaches and in tube labeling. The superiority of this in-capillary approach over the conventional batchwise protocol relies on the reduction of sample and reagent

consumption (fourfold) and its coupling to glycan separation by CE-LIF with no evident resolution degradation. Another positive feature is its automatization and integration, avoiding sample loss and too high variability between analyses and giving access to a high-throughput analysis perspective by using a multiple-channel CE analyzer. Our ultimate goal is to replace the batchwise in-tube protocols with an automated online protocol to allow high throughput and no cross contamination. The present work on in-capillary glycan labeling can be considered the first and solid brick to achieve this goal. The combination of online digestion and online labeling of glycans is envisioned for their subsequent CE-LIF and CE-MS analyses.

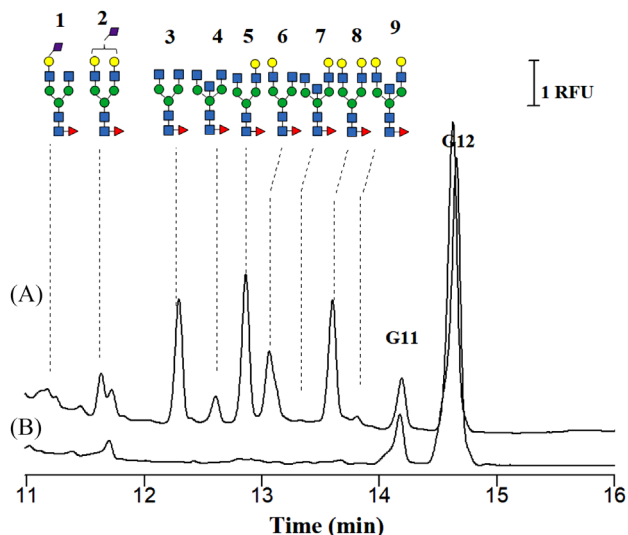


FIGURE 5 In capillary labeling of IgG-released N-glycans (from a 10 $\mu\text{g}/\mu\text{L}$ protein sample diluted twofold after N-glycan release) (a) and a blank without IgG (b). An internal standard APTS - G12 was added to the released glycan solution and the blank solution before injection at a final concentration of 200 mM (the G12 sample also contains traces of maltopentaose APTS - G11). CE conditions: 8 mM NaBH_3CN in 20% HAc (v/v) at 45°C, incubation time 50 min, mol ratio of APTS/Glycans = 29,150 (glycans = 6 μM), plugs volume ratio APTS:Glycans: APTS = 1:2:1. Symbols: \blacksquare , N-acetylglucosamine; \bullet , mannose; \bullet , galactose; \blacklozenge , N-acetylneuraminic acid; \blacktriangle , fucose

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article, and can also be provided by 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.

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