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Lab-in-droplet: From glycan sample treatment toward diagnostic screening of congenital disorders of glycosylation

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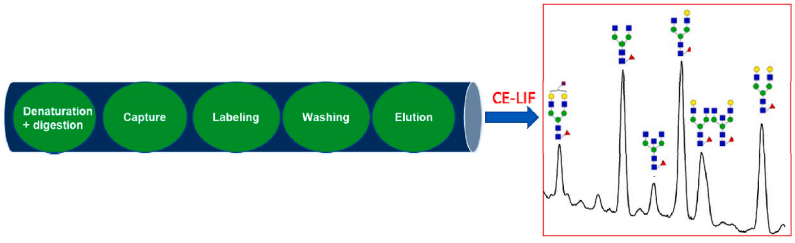
HIGHLIGHTS

- A novel microfluidic droplet-based platform for high performance multi-step glycoprotein sample treatment was developed.
- Positive features not available in previous droplet setups, notably automation, droplet sensing and heating were developed.
- A complex sample treatment protocol feasible so far only in batchwise mode can be converted into a microfluidic version.
- Mapping of N-linked glycans from human sera was made for diagnostic screening of congenital disorders of glycosylation.

GRAPHICAL ABSTRACT



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ABSTRACT

We present in this study a new microfluidic droplet platform, named Lab-in-Droplet, for multistep glycoprotein sample treatment. Several operations are required for the sample treatment of a given glycoprotein to profile its N-glycans. In our case, all preparation steps for the analysis of N-glycans from glycoproteins could be realized in an automatic manner and without cross contamination. This could be achieved through several features that are not met in previous droplet setups, notably full automation, droplet sensing and heating. The magnetic tweezer technology was employed to manipulate (capture and release) coated magnetic beads used as analyte cargos over droplets. Droplets ranging from 1 to 10 μL play the role of confined microreactors, allowing to realize several steps that involve advanced functions such as heating and mixing with organic solvents. A complex sample treatment protocol that has been feasible so far only in batchwise mode can now be converted into a novel microfluidic version. With this Lab-in-Droplet, we can enzymatically release and fluorescently label N-linked oligosaccharides from Human Immuglobulin G and then off-line analyze the labeled glycans by capillary electrophoresis with laser induced fluorescent detection. We demonstrated the superiority of this Lab-in-Droplet over the conventional batchwise protocol, with 10-fold less reagent consumption, 3-fold less time, and 2-fold

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improvement of glycan labeling yield, without degradation of glycan separation profile obtained by capillary electrophoresis. The platform with the developed droplet protocol was applied successfully for mapping N-linked glycans released from human sera, serving for diagnostic screening of congenital disorders of glycosylation.

1. Introduction

Glycosylated proteins represent half the number of all secretory and human cellular proteins. Regular functioning and activity of glycoproteins can be harmed by an altered glycosylation [1,2]. This latter, which may result in unoccupied glycosylation sites, truncated antennae of glycans, unexpected glycosidic linkages or glycan structures as well as changes in the proportion of glycoforms for a given glycoprotein, can be the consequence of a pathological state [3]. Indeed, several glycoproteins have been considered as potential or validated biomarkers for diverse diseases such as cancer, cardiovascular diseases [4], chronic alcohol abuse [5] or congenital disorders of glycosylation (CDG) [6]. CDG in particular represent a family of more than 150 inherited diseases [6,7]. Regarding the best known and most frequent CDG affecting the N-linked glycosylation, two main types are considered: CDG-I where some N-glycosylation sites are unoccupied and CDG-II where only subtle structural changes of glycans occur [6]. For the latter where severe pathology must be diagnosed very early on newborns, analysis of released glycans from glycoproteins is of great interest [4,8].

Recently, capillary electrophoresis (CE) coupled with laser induced fluorescent (LIF) detection has become an established method for glycan analysis [9,10], with the introduction of commercial kits [11,12]. In a standard protocol, released glycans are first fluorescently labeled with 8-aminopyrene-1,3,6 trisulfonic-acid (APTS, a negatively charged fluorophore) and then separated by CE-LIF using acidic background electrolytes (BGEs) containing polyethylene oxide (PEO) or polyethylene glycol (PEG) [13]. To cope with challenges for the detection of low-abundant glycans used as biomarkers, efforts have been made toward glycan enrichment methods based on solid phase extraction [14,15] or electrokinetic phenomena (notably isotachophoresis and large volume sample stacking preconcentration approaches) [16–19] prior to CE-LIF. To further increase the detection sensitivity for CE-LIF of glycans required for diagnosis of glycan-related diseases, we recently developed new BGE conditions that allow much improvement of peak signals and capacity compared to those provided by commercial kits [20].

The forefront sample treatment prior to the CE-LIF profiling of glycans is of extreme importance and may have a crucial impact on the glycan separation efficiency and reproducibility. A typical protocol for such purpose includes glycoprotein denaturation, enzymatic deglycosylation with PNGase F (Peptide-N-glycosidase F, to release asparagine-linked oligosaccharides from glycoproteins), residual protein removal, fluorescent labeling of released glycans with APTS [21], glycan washing and removal of residual fluorophores, and finally elution of labeled glycans for subsequent CE-LIF analysis. The batchwise tube-based approach developed by Guttman et al. that employs magnetic beads as the solid support to realize these steps has become now the gold standard method [22–24], with the recent introduction of a commercial kit [11]. This approach that contains different manual in-vial operations and requires relatively high starting concentrations of glycoproteins has been mostly applied for the purpose of drug discovery and development [11]. Based on this method, PerkinElmer developed recently a microchip-CE platform that transposes the in-vial operations into a 96-well microtiter-plate-based protocol to release and label N-glycans prior to their separation using microchip electrophoresis [25].

In a related context, microfluidics with its inherent advantages of reduced sample and reagents consumption, and shorter reaction time has gained much attention for sample processing [26]. Interestingly, while microfluidics-based sample handling was developed for deglycosylation prior to HPLC separation and/or MS detection of released glycans [27], this miniature approach has never been reported for forefront sample processing for downstream CE-LIF of glycans, to the best of our knowledge. Indeed, glycan sample treatment for CE-LIF requires several steps other than deglycosylation that involve advanced functions (e.g., heating and mixing with organic solvents), which can hardly be down-scaled without any risk of cross contamination between steps. This may be a reason for this delay of development despite the high potential of microfluidics. From our viewpoint, recourse to droplet microfluidics, an emerging branch of microfluidics that allows creation and manipulation of discrete droplets considered as micro-reactors inside microfluidic channels [28], could be a solution to overcome such challenge. Accordingly, we present in this study a new instrument, named Lab-in-Droplet, and its respective protocol for glycan sample treatment in microfluidic droplets, serving as a forefront for CE-LIF of glycans. New functions that have not been developed in previous droplet systems, including droplet fusion with organic solvents, droplet heating and droplet sensing for automation of the protocol, are explored and exploited for protein denaturation, enzymatic release of N-glycans, residual protein removal, fluorescent glycans labeling, washing and elution to provide an automatic process and avoid cross contamination. The system was applied to the N-glycan mapping of human Immuglobulin G (IgG) and then to total N-glycans released from human serum glycoproteins. We exploited the profiles obtained from both a healthy and a CDG patient after the processing with Lab-in-Droplet to serve for diagnostic screening of CDG.

2. Experimental

2.1. Chemicals, reagents and samples

All chemicals for preparation of buffers were of analytical or reagent grade and purchased from Sigma-Aldrich (Lyon, France). Reagents for preparation of glycans (denaturation, digestion, labeling solutions; malto-oligosaccharides (MD ladder), APTS dye, magnetic beads and G3 standard) were obtained from the Fast Glycan Analysis and Labeling kit from Sciex (Villebon sur Yvette, France). Human Immunoglobulin G, PNGase F and sodium cyanoborohydride were purchased from Sigma-Aldrich (Lyon, France). For the preparation of background electrolyte (BGE) solutions, Triethanolamine (TEOA) and citric acid (Cit) were used. The analysed serum samples came from the biochemistry department of Bichat Hospital (Assistance Publique - Hôpitaux de Paris, France). This department is officially accredited (French Committee of Accreditation COFRAC -ISO15189 under the number: 8–3490) and is officially recognized as a "Reference Medical Biology Laboratory" (RMBL) in the field of Congenital Disorders of Glycosylation (CDG). All ethical requirements were met to obtain related official agreements. Informed consents were obtained from the adult representative(s) of all tested individuals. Fluorinated oil containing surfactant was obtained from Inorevia (Paris, France).

2.2. Apparatus and material

The CE experiments were performed with a Beckman Coulter PA800+ system (Sciex, Brea, CA) coupled with a LIF detector ($\lambda_{excitation}$: 488 nm, $\lambda_{emission}$: 520 nm). 32Karat ver 8.0 software (Sciex) was used to carry out the instrument control and the data acquisition. Separations were performed with a fused silica capillary of 50 μm id and 375 μm od from Polymicro (TSP075375, CM Scientific, Silsden, UK), with the total length (L_{tot}) of 60 cm, the effective length (L_{eff}) of 50 cm. Deionized water was produced using a Direct-Q3 UV purification system (Millipore, Milford, MA, USA). The measure of the pH values of buffer

solutions and samples were done with a SevenCompact pH meter (Mettler Toledo, Schwerzenbach, Switzerland). Selection of BGE and buffer ionic strength (IS) calculations were based on simulations with the computer program PhoeBus (Analis, Suarlée, Belgium).

The instrument for droplet handling was developed by Inorevia (Paris, France) according to the criteria and parameters pre-defined by our group (see Fig. S1 in the electronic supporting information (ESI)). It includes a stepper-motor-driven syringe pump with a 250 μL syringe connected to a 3-port selector valve, a magnetic tweezer, two thermal modules and three optical detectors. PTFE tubing is used for all connections. The outlet of the PTFE tubing can be moved into different vials in a vial holder thanks to a robotic arm. Up to 14 vials can be used to load reagents, sample solutions, magnetic beads, oil and for trash reservoir. More details regarding system design can be referred to section 3.1.

2.3. Methods

2.3.1. Fluorescent labelling of standard malto-oligosacharides (MD ladder) in tube

The protocol for fluorescent labelling of MD ladder was adapted from that by Reider et al. [22]. Briefly, 50 μL of beads was added in a 200 μL PCR tube. After removing the supernatant, 10 μL of MD ladder (0.1 mg/mL in deionized water) and 200 μL of acetonitrile (ACN) were added. After mixing, the supernatant was removed. Then, labelling reagents, including 11 μL of 40 mM APTS in 20% AcOH, 1 μL of 1 M $NaBH_3CN$ and 1 μL of NP-40 were added into the sample vial and incubated for 20 min at 60 °C with opened cap. ACN 90% was then added to labeled glycans jammed on magnetic beads while the supernatant containing excess of fluorophore was removed. The elution of glycans captured on magnetic beads was then performed with 20 μL of water.

2.3.2. Enzymatic release and fluorescent labelling of glycoprotein-derived-glycans in batch

10 μL of IgG (10 mg/mL in deionized water) was mixed with 5 μL of the denaturing solution containing 10 μL of D1, 10 μL of D3 and 50 μL of D4 from the 'fast glycan labelling and analysis' kit from Sciex and incubated at room temperature for 7 min. After denaturation, 12 μL of the digestion solution containing 1 μL of PNGase F in 11 μL of D4 was added and further incubated for 20 min at 60 °C. The obtained solution containing released N-glycans was added to a 200 μL PCR tube containing 50 μL of beads without supernatant. The labelling step was then carried out following the protocol described in Ref. [22]. Briefly, labelling reagents (11 μL of 40 mM APTS in 20% AcOH, 1 μL of 1 M $NaBH_3CN$ and 1 μL of NP-40) were added into the sample vial and incubated for 20 min at 60 °C with opened cap. ACN 90% was then added to jam labeled glycans on magnetic beads while the supernatant containing excess of fluorophore was removed. The elution of glycans captured on magnetic beads was then performed with 20 μL of water. This eluent was subsequently analysed with CE-LIF.

For the preparation of N-glycans from human serum samples, the magnetic beads delivered in the 'fast glycan labelling and analysis' kit from Sciex were used for sample purification. The tube containing 200 μL of resuspended magnetic beads was placed in a magnetic stand to allow the beads migrating to the magnet, then the storage buffer was removed. This step was performed every time to remove any liquid from the beads. 10 μL of serum sample (4-fold diluted in water) was added onto the beads. 5 μL of denaturing solution was then added, mixed and incubated at room temperature for 7 min with the vial opened. 11 μL of digestion solution from the 'fast glycan labelling and analysis' kit from Sciex was added to the mixture; and incubated at 60 °C for 20 min in an open vial. 200 μL of ACN was then added, mixed and incubated at room temperature for 1 min. The supernatant was removed, and 9 μL of 40 mM APTS in 20% acetic acid, 1 μL of NaCNBH3 et 1 μL of D4 from the 'fast glycan labelling and analysis' kit from Sciex was added, followed by incubation at 60 °C for 20 min in an open vial. The subsequent washing and elution steps were then carried out as for the case of IgG.

2.3.3. Fluorescent labelling of standard malto-oligosacharrides in droplets

A total of 2.5 μL of magnetic bead suspension (10 mg/mL) was aspirated in the microfluidic droplet device. The magnetic tweezer was then activated to trap the beads and the supernatant was discarded to the trash tube. The trapped beads were then suspended in a 1 μL of MD ladder (0.1 mg/mL in deionized water). 7 μL of ACN was then merged with the droplet containing the beads and the MD ladder. After a 5 min incubation, the supernatant was removed. The beads were then resuspended in a 1 μL droplet of the labelling solution (40 mM APTS in 20% AcOH/1 M $NaBH_3CN$/NP-40 of 9/1/1 v:v ratio). The mixture was incubated for 20 min at 60 °C. This droplet was then merged with 9 μL of ACN to jam labeled MD ladder on beads; and the supernatant droplet containing residual APTS was moved to the trash vial. Elution of captured MD ladder was then performed in 6 μL of water. This eluent was subsequently analysed with CE-LIF. Further information about the microfluidic device parameters for each step can be found in Table 1.

2.3.4. Denaturation, digestion, and fluorescent labelling of glycoprotein derived-glycans in droplets

A total of 2.5 μL of magnetic bead suspension (10 mg/mL) was aspirated in the microfluidic droplet device. The magnetic tweezer was then activated to trap the beads and the supernatant was discarded to the trash tube. The beads were subsequently suspended in a 2 μL droplet of standard IgG (10 mg/mL) in PBS, or serum diluted 4 times in water. The droplet containing the beads and the sample was merged with 1 μL of denaturation solution that contains SDS, DTT and NP-40 at v:v ratio of 1/1/5. After incubation over 7 min, the obtained droplet (3 μL) was merged with 2.4 μL of digestion solution containing NP-40 and PNGase F at v:v ratio of 11/4. The 5.4 μL droplet was incubated during 20 min at 60 °C. The beads were then captured by the tweezer and the solution was recovered in a vial. 1 μL of the recovered solution was then aspirated and the beads were resuspended in it. A droplet of 7 μL ACN was then merged with this droplet. After incubation over 5 min, the beads were retained at the tweezer and the supernatant was removed. The beads were then resuspended in a 1 μL droplet of the labelling solution (40 mM APTS in 20% AcOH/1 M $NaBH_3CN$/NP-40 of 9/1/1 v:v ratio). The mixture was incubated for 20 min at 60 °C. This droplet was then merged with 9 μL of ACN to jam labeled glycans on beads, and the supernatant droplet containing residual APTS was moved to the trash vial. Elution of captured glycans was then performed in 6 μL of water. This eluent was subsequently analysed with CE-LIF. Further information about the microfluidic device parameters for each step can be found in Table 1.

2.3.5. CE-LIF of APTS-glycans and oligosaccharides

Analyses of APTS-labeled glycans or malto-oligosaccharide standards were carried out with our recently developed BGE composed of TEOA/citric acid (IS of 150 mM, pH 4.75) [20]. These separations were done using a fused-silica capillary of 50 μm id, with L_{tot} of 60 cm and 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, 0.1 M NaOH for 5 min, water for 5 min and the BGE for 30 min prior to first use. Note that no HCl deactivation was needed, because there was no capillary coating in our protocol; and with our new BGE composition at a high ionic strength we could suppress well and reproducibly the surface charge of the capillary. Inter-run rinsing was done with the BGE for 5 min.

3. Results and discussion

3.1. Design of the microfluidic droplet system

The Lab-in-Droplet system for glycoprotein sample treatment, illustrated in Fig. 1, was developed and optimized to bring new features that were not feasible in any previously reported systems using droplets in oil. First, automatic detection and control of droplet position were

Table 1
Protocol for sample droplet glycans. RT: room temperature.

Step	Step in droplet	Droplet size	Optimized parameters
Denaturation	Droplet of beads with mixing	2.5 μL	
	Capture of the beads by the tweezer	2.5 μL	
	Trash the supernatant	2.5 μL	
	Droplet of sample	2.0 μL	
	Resuspension of the beads in the droplet	2.0 μL	
	Merging between sample and denaturation solution	3.0 μL	
	Incubation at RT	3.0 μL	**Droplet speed:** 600 μL/min **Incubation time:** 7 min
Digestion	Merging between sample and digestion solution	5.4 μL	
	Incubation at 60 °C	5.4 μL	**Droplet speed:** 600 μL/min **Incubation time:** 20 min
	Capture of the beads by the tweezer	5.4 μL	
	Recovery of the sample	5.4 μL	
Capture of the glycans	Droplet of sample recovered previously	1.0 μL	
	Resuspension of the beads in the droplet	1.0 μL	
	Merging between sample and ACN	8.0 μL	
	Incubation at RT	8.0 μL	**Droplet speed:** 600 μL/min **Incubation time:** 5 min
	Capture of the beads by the tweezer	8.0 μL	
	Trash the supernatant	8.0 μL	
Fluorescent labeling	Droplet of labelling solution	1.0 μL	
	Resuspension of the beads in the droplet	1.0 μL	
	Incubation at 60 °C	1.0 μL	**Droplet speed:** 300 μL/min **Incubation time:** 20 min
Washing	Merging between sample and ACN	8.0 μL	**Fusion flowrate:** 800 μL/min
	Incubation at RT	8.0 μL	**Droplet speed:** 600 μL/min **Incubation time:** 3 min
	Capture of the beads by the tweezer	8.0 μL	
	Trash the supernatant	8.0 μL	
Elution	Droplet of H2O	6.0 μL	
	Resuspension of the beads in the droplet	6.0 μL	
	Incubation at RT	6.0 μL	**Droplet speed:** 500 μL/min **Incubation time:** 3 min
	Capture of the beads by the tweezer	6.0 μL	
	Recovery of the sample	6.0 μL	

implemented thanks to an array of optical detectors positioned along the tubing containing oil and droplets. Along the tubing, three optical detectors were installed to detect a change in the optical signal when the meniscus of a droplet passes in front of it, allowing to precisely monitor the position of a given droplet. The first detector was placed before the magnetic tweezer (acting as a magnet), the second between the magnetic tweezer and the first thermal module, and the last after the second thermal module. The magnetic tweezer can be therefore automatically activated (allowing the clustering of magnetic beads inside the tubing) or deactivated (allowing the re-suspension of the beads into droplets inside the tubing) upon passage of the droplet containing magnetic beads that leads to a change in optical signal. Second, a thermostated compartment made of two Peltier plates and integrated with thermal sensors were added in order to allow control of temperatures during the relevant incubation steps of the process. With our design, we don't heat up as a "bulk" the whole system as for droplet PCR, but rather allow more flexibility to move one drop onto a thermal module and the possibility to work with fixed temperatures or with thermal cycling when needed. Temperature ranging from 23 °C up to 92 °C could be established in approximately 1–10 s, with a precision of ± 1 °C. Each heating module can be used independently if only one preset temperature is desired. Both can be activated if more precise temperature control is needed. This allows working with microfluidic-droplet-based sample processing at elevated temperatures. Third, the system was designed to allow pre-mixing of magnetic beads in vials before aspirating them into the tubing in order to maintain high homogeneity of beads in droplets. To avoid sedimentation of magnetic beads in the vial, which was the case in previous systems [29–32], the beads were homogenized in the vial by a series of back-and-forth pushing at a flow rate of 2000 μL/min prior to aspiration of the droplet containing magnetic beads into the tubing. Furthermore, by carefully and accurately tuning the distance between droplets that is monitored with the optical sensors, precise merging of two separate droplets in tubing was made possible. These unprecedented features allow to carry out complex sample processing that has not been possible so far in microfluidic formats. To implement all basic and advanced droplet operations, the system relies on a syringe pump connected to a 3-port selector valve and the droplet handling manifold fixed onto a 'head' block that is mounted on a two-dimensional translation robot (Fig. 1). Droplet sensing, fusion and heating, as well as capture and release of magnetic beads into droplet were indeed implemented on the 'head' block. By pulling oil into the syringe with valve in position 1, the syringe can be refilled to ensure sufficient oil quantity to perform fluidic operations. By advancing the piston of the syringe with the valve in position 2, the syringe can be rinsed with fresh oil, allowing the removal of any air bubble or unwanted droplets/beads. Finally, in valve position 3, oil can be dispensed into or aspirated from the manifold for fluidic operations. The instrument is controlled by a computer, and a purpose-made graphical user interface was developed to allow manual intervention, display of the optical signals of the three detectors, setting and monitoring of the temperature of the thermal modules and implementation of automated protocols.

3.2. Development of microfluidic droplet protocol for glycoprotein sample treatment

3.2.1. Microfluidic operation and optimization

The Lab-in-droplet protocol developed for the glycoprotein sample treatment is illustrated in Fig. 2. In the 'bead capture' operation (Fig. 2A) required for the steps of glycan capture on magnetic beads, labeling of glycans, washing of labeled glycans and elution of labeled glycans, the magnetic tweezer was activated when a droplet containing magnetic beads passed in front of it in order to cluster the beads. The droplet without magnetic beads was then either recovered or trashed. In the 'bead resuspension' operation (Fig. 2B), occurring in different media (APTS solution for glycan labeling, ACN medium for glycans precipitation on beads, or water for elution of glycans), the tweezer is deactivated to allow the target droplet containing the suspension liquid passing through the optical sensor, and resuspend the beads in a synchronized manner. In this 'bead resuspension' operation, it is critical that the droplet fully englobes the aggregated beads. One big challenge when automatically handling small droplets (1 μL) was to monitor and confirm the presence of the target droplet in front of the tweezer prior to release of magnetic beads. The risk of having magnetic beads released in oil outside the target droplet occurred when the viscosity of the liquid inside the droplet varied. This was particularly the case when using ACN

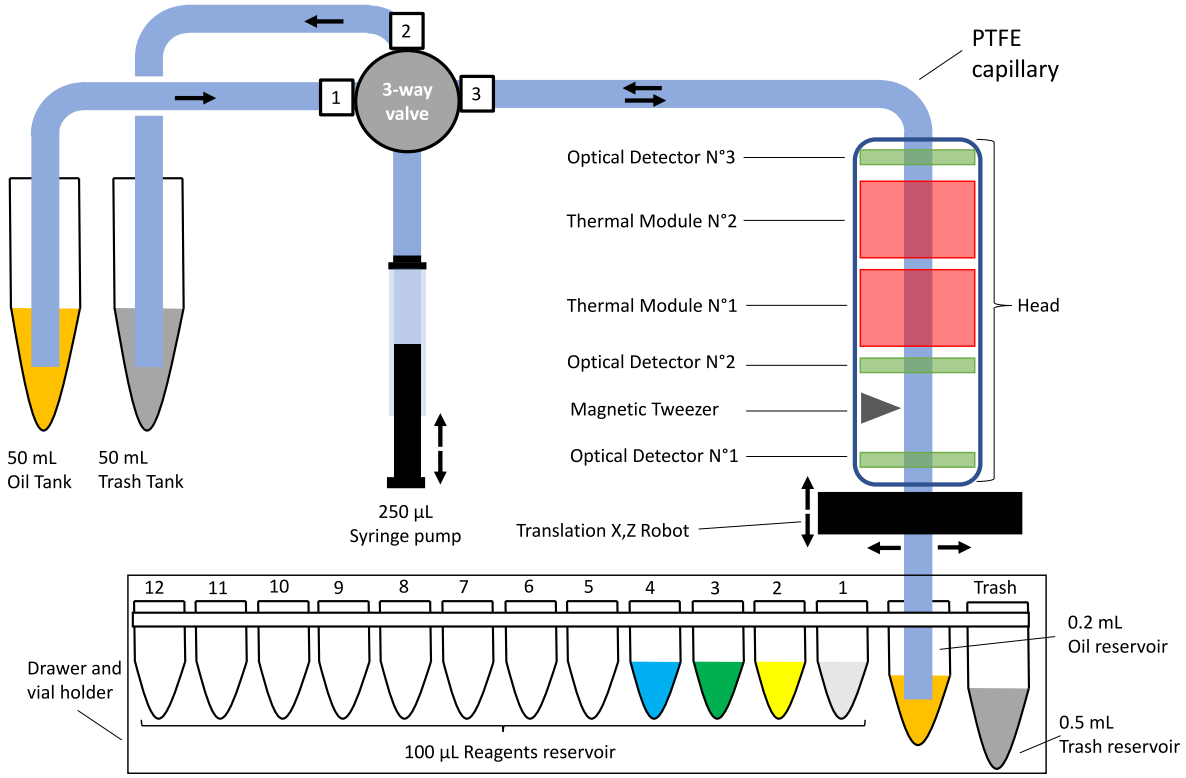

Fig. 1. Schematic drawing of the Lab-in-Droplet system (a prototype named Magelia® by Inorevia) used in our work, adapted from Magelia® user manual.

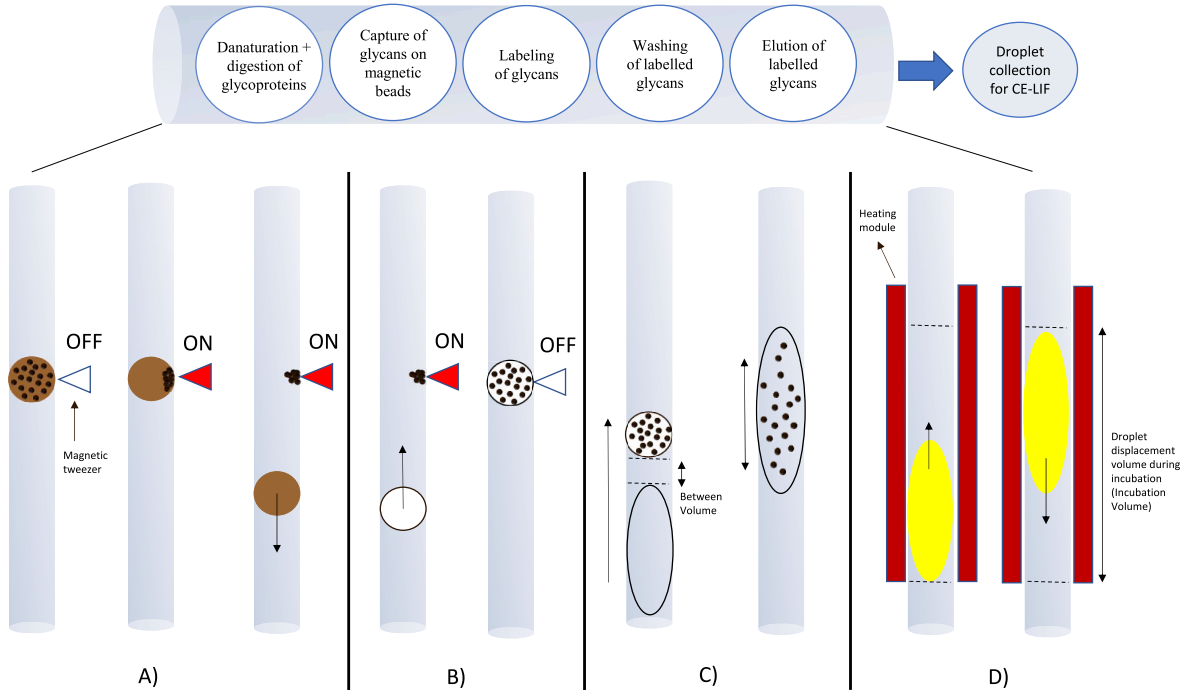

Fig. 2. Basic operations performed with our Lab-in-Droplet system in order to carry out the glycoprotein sample treatment: A) Bead capture, B) Bead resuspension, C) Droplet fusion and D) droplet incubation.

medium that has a lower viscosity than water and other aqueous solutions. Optimization and adjustment of the distance between the magnetic tweezer and the arriving droplet prior to releasing magnetic beads from the tweezer was therefore needed. The next operation required for this protocol is the droplet merging (Fig. 2C), which is crucial at almost all steps of the protocol, in order to mix the sample droplet with the denaturation, digestion, or labeling one. Droplet merging is also required to fuse ACN with the glycan-containing droplet to aggregate glycans onto the magnetic beads. To trigger droplet merging, the first droplet was brought to the optical detector 1 (see Fig. 1) for identification, and then delivered to the vicinity of the tubing outlet prior to aspiration of the second droplet. To prevent the unwanted premature

fusion during the merging step that would lead to loss of the sample, the small oil volume between the two droplets needs to be carefully tuned. This premature fusion was seen to occur more often when magnetic beads were present in the leading droplet. The between-droplet volume was therefore optimized and the range of 0.5–0.7 μL was found optimal to avoid such a problem. By pulling the two droplets back into the tubing at a sufficiently high flow rate, the desired merging of two droplets having different viscosities (*e.g.*, the ACN droplet and the sample one containing glycans and magnetic beads) could occur. The droplet merging happens indeed thanks to the intrinsic difference between the speeds of two consecutive droplets having different viscosities and/or interfacial tensions between oil (containing the surfactant) and the droplet in a straight channel [33]. The pulling flowrate must be rigorously tuned to allow successful merging at a desired position along the tubing. A high flow rate of 900 μL/min was found to successfully trigger droplet fusion without breaking droplets during their movement. The last operation concerns the droplet incubation, in which the target droplet (*e.g.*, the droplet containing glycans and fluorescent labeling agent) is moved back and forth within the thermal modules. This is carried out via a series of aspiration and then dispense of a preset volume of oil to 'shake' the droplet in the tubing. During this step, thermal modules can be turned on if needed. To achieve the best mixing efficiency during glycoprotein denaturation, digestion as well as glycan labeling and washing, we put our efforts to optimize the incubation volume, *i.e.*, the displacement volume of oil being aspirated and dispensed, the oil flowrate and the time of incubation. Our results revealed that the incubation (displacement) volume equivalent to that of the droplet allowed the best homogenization of magnetic beads inside the droplet (Fig. S2 in ESI). From the studies on the hydrodynamics of droplet microfluidics [34–36], an increased flow rate would lead to better mixing behavior, thus allowing to shorten the incubation duration. Indeed, in the classical protocol, the labeling step requires an incubation in tube for 2 h at 60 °C. To shorten this incubation time, several flowrates were therefore tested to achieve the best efficiency of oligosaccharide labeling with APTS, (see Fig. S3 in ESI). The flowrates of 600 μL/min without the use of thermal modules, and 300 μL/min with the assistance of thermal activation (60 °C) were found to be optimal without provoking droplet breaking during this 'shaking' incubation. The droplet was often deviated from its starting position after the back-and-forth incubation at high flowrates due to inevitable penetration of dissolved air into the tubing. The droplet could even go out of the visualization zone of the detector D2, resulting in the loss of droplet detection signal and cease of the system. This problem was more pronounced when the droplet heating (up to 90 °C) was activated during incubation. To avoid this problem, the starting position of the droplet prior to incubation (for example incubation of released N-glycans with APTS, see Fig. 2D) needs therefore to be optimized and adjusted for each droplet matrix, *i.e.*, moving the initial droplet position upward if the droplet tends to deviate downwards from its starting position, as for the case of droplets containing ACN.

3.2.2. Optimization of the glycoprotein treatment protocol

The full protocol for glycoprotein sample treatment, including N-glycoprotein denaturation and digestion, capture of released glycans on magnetic beads, labeling of glycans, washing of labeled glycans and elution of labeled glycans, was developed step by step in droplets and is summarized in Table 1. The glycan capture and labeling steps were optimized first, using oligosaccharide standards that are similar to glycans without the need for forefront denaturation and digestion. To capture and accumulate efficiently all glycans or oligosaccharides onto negatively charged surface of magnetic beads, a solution of 90% ACN is required in tube-based protocols [23,37]. This however is a big challenge in droplet microfluidics when a sample droplet (1 μL) has to be fused with a large plug of ACN (9 μL) to reach this high ACN ratio. The omnipresence of ACN in the merged droplet provoked clustering of magnetic beads at the bottom of the droplet (see Fig. S2 in the ESI). At the same time the droplet became more fragile due to its size and was prone to breaking. To solve this problem, the amount of ACN to be merged with the droplet sample (1 μL) was reduced from 9 μL to 7 μL (i.e., reducing the ACN ratio from 90% to 87.5%); and a flowrate of 600 μL/min was used for back-and-forth agitation to minimize beads clustering. The capture efficiency and subsequent labeling of oligosaccharides were found unaffected by this ACN ratio reduction thanks to the better mass transfer offered by microfluidics. To remove the excess of fluorophores which is detrimental to subsequent CE-LIF, washing was performed after the labeling step. This indeed involved merging of the droplet containing labeled oligosaccharides and circulating magnetic beads with a large ACN droplet, using the same principle as for the step of glycan capture on beads. It is shown in Fig. 3 the electropherograms of in-droplet-labeled oligosaccharides with different numbers of subsequent washing steps. With no washing, the huge signal of residual fluorophores, observed as the baseline drift over the whole electropherogram, overlapped the peaks of oligosaccharides, which is detrimental for their detection (Fig. 3A). With two washings using two consecutive droplets containing ACN, oligosaccharides were lost and therefore not observed in the electropherogram (Fig. 3C). A compromise was found with one washing droplet only, where residual fluorophore and by products were well removed (reflected by a flat baseline) while the peak zones for oligosaccharides (from 13 min) were not disturbed (Fig. 3B). It is important to note that a tube-based protocol needs at least 5 washing steps to reach an equivalent quality of washed and labeled oligosaccharides achieved with one washing using the Lab-in-Droplet. The droplet protocol development was then extended to N-glycoprotein denaturation and enzymatic digestion, using standard human IgG. In this case, the sample droplet containing IgG was first fused with another one containing a mixture of denaturing and digesting reagents, followed by droplet incubation to release N-glycans from IgG. When adapting the enzyme quantity used for the tube-based method (*i.e.*,

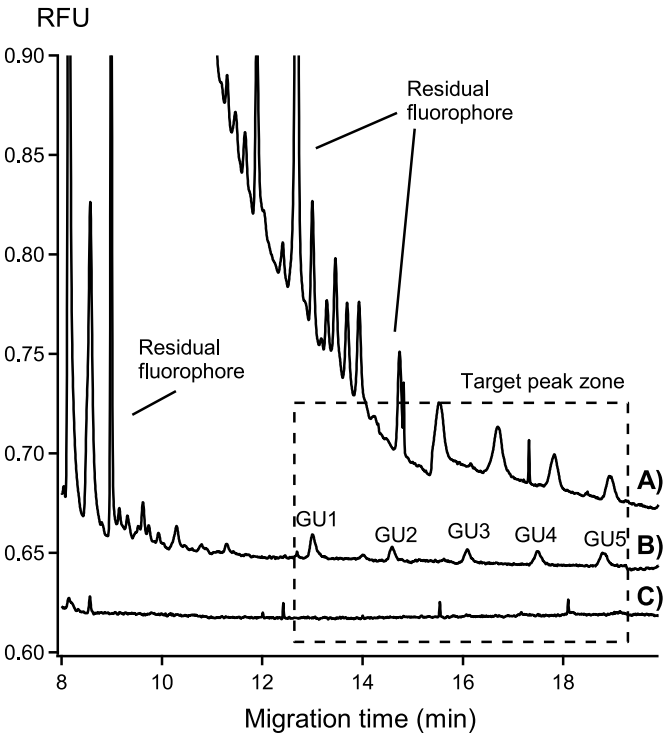

Fig. 3. Electropherograms of in-droplet-APTS-labeled oligosaccharides: A) Without washing step, B) With 1 washing step and C) With 2 washing steps. CE-LIF conditions: fused silica capillary, 50 μm i.d., 50/60 cm, hydrodynamic injection at 50 mbar for 10 s, high voltage of −25 kV. BGE: Triethanolamine and citric acid at ionic strength 150 mM and pH 4.75. LIF detection at $\lambda_{excitation}$ of 488 nm and $\lambda_{emission}$ of 520 nm.

commercial PNGase F enzyme accounting for 8.3% of the digestion solution volume) to the in-droplet IgG digestion, the glycans' signals could hardly be detected with subsequent CE-LIF analysis (see Fig. S4). An increase in PNGase F quantity by 4-folds allowed clear visualization of all IgG glycans' peaks, confirming successful release of glycans from IgG. Higher enzyme concentrations were also tested but no further improvement of glycans' peak intensity was observed (data not shown). While the translation of tube-based conditions to droplet ones to release glycans from IgG was straightforward thanks to the absence of magnetic beads in these two steps, the challenge resided in the subsequent coupling to the next step of glycan capture on beads. The initial volume of the mother droplet containing released glycans had to be reduced from 5.4 μL (see Table 1) to 1 μL prior to fusion of this daughter droplet with a large ACN droplet to reach a final volume of up to 10 μL (*i.e.*, the maximum volume that can be handled by Lab-in-Droplet). This volume reduction was implemented via precise handling of different droplet manipulations, including delivery of the mother droplet (5.4 μL) out of the tubing without ejecting oil and retaining of a daughter droplet (1 μL) at the tubing's extremity.

3.2.3. Method validation

Efforts were then made to validate each step in the droplet-based protocol using the optimized parameters for glycoprotein denaturation, digestion, glycan labelling and elution. This is very challenging. Indeed, to obtain results for the validation of each droplet-based step, the steps onwards had to be continued with in-tube batchwise operations, which were unfeasible for some of the considered steps. Accordingly, the outlet micro-droplet to be validated had to be collected after each step, transferred, and diluted in vials to continue the subsequent steps in-tube, prior to the second collection for CE-LIF analysis. All these manual collections and transitions may lead to operational errors and additional variability that should be considered when interpreting the obtained validation data. This could lead to results that could not perfectly reflect the repeatability (for instance) of each step and consequently underestimate largely the validation results. It is shown in Fig. S5A in the ESI the results from triplicates of each step. The variations of CE-LIF signals were better than 22% for the step of glycoprotein denaturation/digestion, whereas they were better than 15% for the step of glycan labelling in droplets. Interestingly, the signal variations for the triplicate of the whole protocol in droplets were around 24%, which are only slightly higher than those obtained with each single step. This demonstrated that the main source of errors could come from the manual intervention for outlet droplet collection and dilution (for subsequent in-tube continuation and/or CE-LIF analysis), and not from the automated in-droplet protocol itself. The relatively elevated RSD values (up to 24%) deem acceptable, considering that the variations of signals obtained with CE-LIF analysis are the accumulation of errors from several operations, including those from the droplet protocol, electrophoresis analysis, as well as manual in-tube sample collection and dilution. The ratios between the target glycan peak signal and the total one of all principal peaks, obtained with both microfluidic droplet and in-tube batchwise protocols, were compared (Fig. S5B in ESI). No significant variations were observed for all tested peaks regardless of the protocol used, demonstrating equal sample treatment performances for both in-tube and droplet protocols for all glycans. This is very important for non-biased glycan analysis serving for the glycan-defected disease diagnosis purpose. The migration times obtained with CE-LIF of glycans from the droplet protocol showed no significant variations (RSD % < 0.3%, Fig. S5C in ESI), allowing identification of glycan peaks over runs.

3.3. Performance of Lab-in-droplet

The Lab-in-Droplet with the full optimized protocol was then used to release and fluorescently label N-glycans from human IgG, and the CE-LIF profile of IgG glycans was compared with that obtained with conventional tube-based protocol (Fig. 4). These IgG glycans are known to

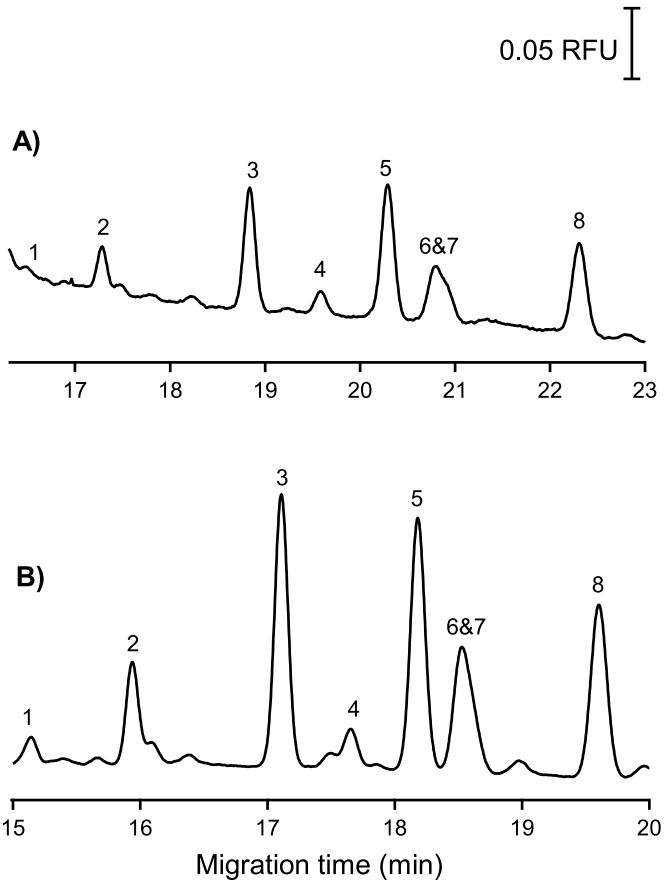

Fig. 4. Electropherograms of APTS-labeled N-glycans released from standard human IgG, using (A) Lab-in-Droplet and B) conventional tube-based protocol. CE conditions as in Fig. 3.

be mainly bi-antennary complex type with low terminal sialylation (5–10%), highly core-fucosylated (>92%); with a small proportion containing a bisecting GlcNAc (11%) [38]. Similar glycan profiles for IgG, in terms of peak height ratios between any two peaks among the 8 abundant ones, were achieved (Fig. 4). This result indicates that the glycan release and labeling steps in the microfluidic droplet format is equally performant for all glycans, which is of extreme importance for non-biased glycan analysis. By referring to similar peak patterns assigned for human IgG already reported with the commercial tube-based glycan treatment and CE-LIF kits [11,39], we could assign the corresponding N-glycan structures to the major peaks obtained with our droplet approach. While there was a 5-fold sample dilution induced by our droplet protocol compared to the tube-based method, we observed only a 2-fold decrease in peak height when comparing the glycan profiles obtained from droplet-based and tube-based protocols (Fig. 4A vs B). Note that the profiles are slightly different for Fig. 4A and B, especially for the peak height ratio of peaks 3 and 5 in the electropherograms. This slight difference may be due to the two different ways of sample preparation (with and without dilution for Lab-in-droplet and in-tube protocols, respectively) prior to CE analysis. The amounts of reagents were reduced by up to 10 folds for Lab-in-Droplet. The droplet protocol is also much shorter (1.3 h) and fully automated, compared to the time required for conventional manual batchwise procedure (2.5 h). To further highlight the superiority of our approach, the performance of Lab-in-Droplet for glycan sample processing was then compared with those achieved with previous methods for the same purpose (Table S1 in the ESI). Among sample treatment methods compatible with CE-LIF of APTS-labeled glycans, our approach offers the shortest duration, full automation, least working sample volumes and no risk of contamination

with external environments. Note that for purpose of diagnostics of glycan-defected diseases, the relative ratios of target glycans in comparison to the total signal of major glycan peaks in the same CE-LIF electropherogram attract more attention than absolute quantification of a particular glycan. The Lab-in-Droplet system is therefore perfectly matching this requirement of glycan profile screening, as it offers equal sample treatment performance for all glycans for non-biased glycan analysis.

For CDG screening via N-glycan analysis, CE-LIF has not been listed yet as a gold method but attracts particular attention and holds great potential thanks to its high performance for glycans separation [6]. In order to showcase the applicability of our Lab-in-Droplet as a forefront for CE-LIF for the CDG diagnostics, total N-glycans from three serum samples, from two CDG-affected individuals and one healthy person (control) were released and labeled with Lab-in-Droplet and analysed with CE-LIF (Fig. 5). Different peaks from highly probable normal biantennary N-glycans were obtained for the control serum from a healthy person (peaks a, b in trace A). The glycan profiles are different for the CDG individual with a known quasi-total deficiency in galactosylation (B4GALT1-CDG) leading to N-glycans with truncated antennae (Trace B), and for the CDG patient with deficiency in GlcNAcylation of one of the 2 antennae of N-glycans (MGAT2-CDG, trace C). It is worthy to note that only human serum samples with confirmed CDG probability were used in this test in order to provide accurate evaluation on the performance of the new instrumental concept. The peak patterns obtained with our droplet protocol were similar to those obtained with the conventional tube-based method (see Ref. [20]), demonstrating the applicability of our Lab-in-Droplet approach for the analysis of glycans from biofluids. Note that the relative fluorescent signal scales for Figs. 4 and 5 are different because the experiments were done at different periods. Direct comparison of the signal intensities in Fig. 4 and those in Fig. 5 is therefore not considered. Regardless of the sample treatment method used (droplet or batchwise), very typical and distinct profiles were obtained for the CDG patient vs healthy person, indicating a high potential for diagnosis of CDG with N-glycan profiling by CE-LIF. At the present stage of the work using our newly developed BGE for CE-LIF of glycans, prediction of glycan structures for human serum samples (Fig. 5) with high precision is not yet possible with the existing software for this purpose.

4. Conclusion

Our novel microfluidic droplet system has been successfully developed for the multiple-step sample treatment of glycoproteins prior to glycan profiling. New approaches in instrumentation and methodology are proposed to this purpose and challenging operations were performed in droplets, using a variety of complex reagents and media. N-glycans from standard human IgG and human serum were successfully released, fluorescently labeled, washed and eluted with this droplet approach for subsequent analysis by CE-LIF. With Lab-in-Droplet, the glycan fingerprints were found similar to those obtained by traditional or macroscopic strategies. At the same time, a signal gain by 2-folds, a reduction of operation time by 2-folds, and a reduction of required reagent amounts by 10-folds could be achieved. Analysis of a large cohort of CDG samples with a more mature version of Lab-in-Droplet under a clinical context will be envisaged in order to validate its potential for the diagnostic purpose. While the inaugural application of Lab-in-Droplet has been made for glycan analysis, this platform could be extended to other biological applications using magnetic beads functionalized with different ligands (e.g., antibodies for immunocapture or immunoassays in droplets). Thanks to automation and miniaturization that we achieved with droplet microfluidics, higher throughput can now be envisioned.

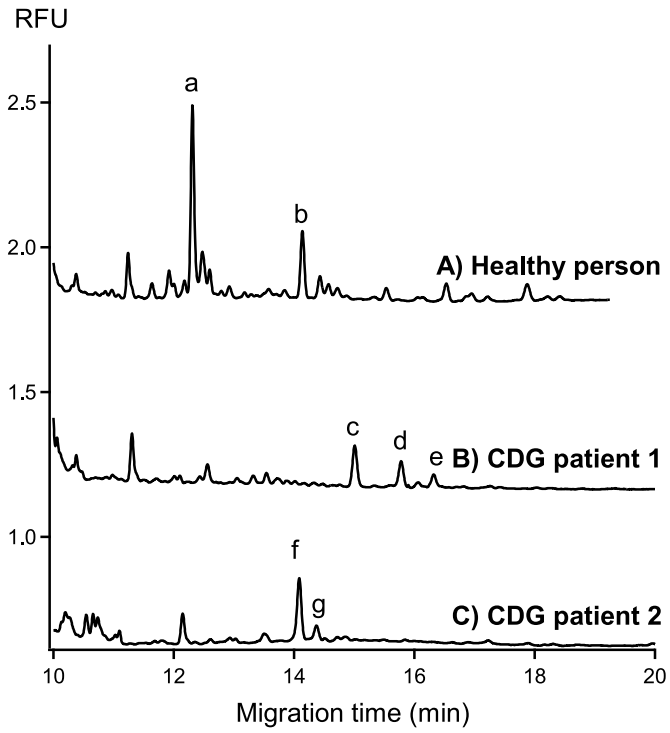

Fig. 5. Electropherograms of APTS-labeled N-glycans released from human serum samples using Lab-in-Droplet. A) Healthy person (control) with peaks a and b from highly probable normal bi-antenated N-glycans; B) CDG patient with a known quasi-total deficiency in galactosylation (B4GALT1-CDG) leading to N-glycans with truncated antennae (peaks c, d, e); and C) CDG patient with deficiency in GlcNAcylation of one of the 2 antennae of N-glycans (MGAT2-CDG) (peaks f, g). CE conditions as in Fig. 3

CRediT authorship contribution statement

Théo Liénard–Mayor: Methodology, Validation, Investigation, Writing – original draft. **Camille Bricteux:** Methodology, Validation, Investigation. **Amel Bendali:** Resources, Methodology, Validation. **Nguyet-Thuy Tran:** Resources, Methodology, Validation. **Arnaud Bruneel:** Resources, Methodology, Validation. **Myriam Taverna:** Investigation, Methodology, Supervision, Writing – review & editing. **Thanh Duc Mai:** Project administration, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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.

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Appendix A. Supplementary data

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