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**Author:** Heemskerk, A.A.M.  
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Chapter 2

Coupling porous sheathless interface MS with transient-ITP in neutral capillaries for improved sensitivity in glycopeptide analysis

Anthonius A. M. Heemskerk, Manfred Wuhrer, Jean-Marc Busnel, Carolien A. M. Koeleman, Maurice H.J. Selman, Gestur Vidarsson, Rick Kapur, Bart Schoenmaker, Rico J.E. Derks, André M. Deelder, Oleg A. Mayboroda

Abstract

Immunoglobulin G antibodies are modulated in their function by the specific structure of the N-glycans attached to their Fc portions. However, the glycosylation analysis of antigen-specific IgGs is a challenging task as antibody levels to a given antigen only represent a fraction of the total IgG levels. Here, we investigated the use of a transient-isotachophoresis (t-ITP) - mass spectrometry method for highly sensitive immunoglobulin G1 (IgG1) glycosylation profiling as a complementary method to a high throughput nano-reverse phase liquid chromatography-mass spectrometry (nano-RPLC-MS) method. It was found that t-ITP-CZE using neutrally coated separation capillaries with a large volume injection (37% of capillary volume) and interfaced to mass spectrometry with a sheathless porous sprayer yielded a 40 fold increase in sensitivity for IgG1 Fc glycopeptide analysis when compared to the conventional strategy. Furthermore, the glycoform profiles found with the t-ITP-CZE strategy were comparable to those from nano-RPLC-MS. In conclusion, the use of the highly sensitive t-ITP-CZE-MS method will provide information on IgG Fc glycosylation for those samples with IgG1 concentrations below the limits of detection of the conventional method.
1 Introduction

Human immunoglobulin G (IgG) molecules contain two heavy chains and two light chains forming together 2 Fab (fragment antigen binding) portions and 1 Fc (fragment crystallizable) portion. Four subclasses of human IgG exist (IgG1, IgG2, IgG3 and IgG4) named in the order of decreasing concentration in human blood [1]. The Fc portions of human IgG contain a conserved N-glycosylation site at Asn 297 of the CH2 domains of their heavy chain. N-glycosylation is important for modulation of IgG activity and is known to be dependent on age, gender as well as on physiological and pathological conditions of an organism [2-9].

Consequently, the analysis of Fc glycosylation of antigen-specific affinity-purified IgGs provides insight into the (dys-)regulation of IgG glycosylation features in health and diseases. For example, we have recently shown that IgG1 directed against human vaccines shows a transient increase of galactosylation and sialylation within the first couple of months after vaccination [10]. In rheumatoid arthritis, pathogenic anti-citrullinated protein antibodies isolated from the inflamed joint of rheumatoid arthritis patients exhibited low galactosylation and sialylation which is interpreted as a pro-inflammatory glycosylation profile [11]. Finally, it has been shown that low core-fucosylation is a characteristic feature of pathogenic IgG1 allo-antibodies produced by pregnant women against platelet antigens of the fetus [5]. These antibodies are transported via the placenta and can destroy the fetus’ platelets, which results in petechiae, major organ bleedings or even intracranial hemorrhages (Fetal/neonatal allo-immune thrombocytopenia; FNAIT). Thus, low core-fucosylation may represent an important pathogenic factor promoting platelet destruction as it is known to lead to enhance ADCC [12].

A number of analytical techniques have been developed for the analysis of protein glycosylation in general, and for the characterization of IgG glycosylation in particular. Commonly used strategies for mass spectrometric IgG glycosylation profiling in proteolytic digests are reversed phase and hydrophilic interaction liquid chromatography coupled to mass spectrometry (RPLC-MS and HILIC-MS) and direct mass spectrometric analysis of glycopeptides by MALDI-MS [13, 14]. In recent years capillary electrophoresis mass spectrometry (CE-MS) has been applied for the analysis of glycopeptides from not only IgG but also erythropoietin proteolytic digests [13-16].
We have developed a robust, high-throughput nano-RPLC-MS method for IgG N-glycosylation profiling [6]: human polyclonal IgGs are captured from 2 µl of plasma or serum by protein G-Sepharose™ beads (GE Healthcare, Uppsala, Sweden) and cleaved overnight with 200 ng of trypsin [17], followed by nano-LCTOFMS analysis of glycopeptides with a lower limit of detection of approximately 870 attomol [6].

The sensitivity of the currently available glycoanalytical methods [6, 13] are found to be sufficient for detailed analysis of total IgG Fc glycosylation from sub-micro liter volumes of plasma or serum. These same methods, however, fall short if applied for the analysis of antigen-specific IgG, allowing the analysis of patient samples with high antigen-specific IgG titers only. Using larger serum or plasma volumes to be able to capture sufficient amounts of the pathogenic auto- or allo-antibodies is not always possible due to limited sample amounts that are available for a single assay as multiple assays are likely required from the same precious sample.

Therefore, we decided to set up a highly sensitive IgG Fc glycosylation assay for the analysis of tryptic Fc glycopeptides on the basis of a previously characterized transient-isotachophoresis (t-ITP) separation strategy. This strategy performs electrophoretic separation in neutrally coated capillaries with porous sheathless interfacing to a mass spectrometer[18, 19]. Similar analytical strategies have previously provided significant improvements in proteomic analysis of various samples [20, 21].

2 Experimental

All analyses were carried out using a Beckman Coulter PA800 plus (Brea, California USA) coupled to a MaXis 4G UHR-TOF from Bruker Daltonics (Bremen, Germany) via a porous sheathless interface designed by Mehdi Moini [18], and now in development by Beckman Coulter. The mass spectrometer was tuned with highest sensitivity in the m/z 700 to 1600 region to register all IgG derived tryptic Fc glycopeptides. The mass spectrometer was operated in positive ionization mode and acquired data in the mass range from m/z 400 to 2900 with a spectra rate of 1 Hz. The permanent capillary coating, currently in development by Beckman Coulter, consisted of a bi-layer with the outer surface based on polyacrylamide. The background electrolyte consisted of 10% acetic acid and the sample buffer/leading electrolyte of 100 mM ammonium acetate pH 4. 37% of the total capillary volume was filled with the sample and 25 kV was applied across the capillary for separation. Concomitantly to the voltage application, a pressure of 1 psi was applied at the capillary inlet to obtain a flow sufficient for stable electrospray
(approximately 8 nl/min). All chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated. Every sample run was internally calibrated using polysiloxane clusters which were present in the mass spectra due to the open source design and the nanoflow regime.

3 Results and Discussion

A tryptic digest of total human plasma IgG purified by protein-G was analyzed, and in accordance with previous observations based on analysis by nano-RPLC-MS [6] 18 IgG1 glycoforms could be determined as both [M+2H]2+ and [M+3H]3+. Structural assignment of the detected glycoforms was performed on the basis of literature knowledge of IgG N-glycosylation [22-26]. For the considered glycopeptides, the t-ITP-CZE system did not show a separation on the basis of the peptide moieties but rather on basis of the attached glycan (Figure 2-1), while glycopeptides from the different IgG subclasses were found to co-migrate (data not shown). This is in contrast to nano-RPLC-MS where a complete separation of the three subclass-specific glycopeptide clusters was observed on the basis of minor differences in peptide sequence [6].

Figure 2-1: (A) Base peak electropherogram of a tryptic digest of total IgG purified from human serum; (B) extracted ion traces of the high concentration IgG1 glycopeptide species including assignment; (C) extracted ion traces of the low concentration IgG1 glycopeptide species including assignment. Blue square, N-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, N-acetyleneuraminic acid.
A comparison of the nano-RPLC-MS [6] and t-ITP-CZE-MS methods was performed using 250 nl of the protein G purified plasma IgG digest. The comparison was performed using a total IgG digest opposed to real patient samples as the samples were only obtained in very low quantities which were insufficient to perform the appropriate comparisons. The IgG digest was used undiluted for nano-RPLC-MS but was diluted a factor 20 for the t-ITP-CZE-MS method to prevent detector saturation. Normalized intensities of IgG1 glycoforms were determined by scaling the peak area of each peak to the total obtained peak area for all detectable IgG1 glycoforms. It was found that the relative abundances of the major glycopeptides were very similar for the two methods (Table 2-1). Subsequently, the sample was analyzed on 3 consecutive days to determine the inter-day reproducibility of the t-ITP-CZE-MS method for larger studies. The inter-day reproducibility in the t-ITP-CZE-MS method showed to be less than that observed for the nano-RPLC-MS (Table 2-1). In view of the fact that this technique allows a significant increase in sensitivity for glycosylation analysis, the observed inter-day reproducibility is certainly acceptable.

Table 2-1: Comparative analysis of a standard IgG1 glycopeptide samples by t-ITP-CZE-MS and RPLC-MS. SD, standard deviation; RSD, relative standard deviation

<table>
<thead>
<tr>
<th>Glycan code ^a\</th>
<th>Compound (m/z ^b)</th>
<th>t-ITP-CZE-MS</th>
<th>RPLC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[M + 2H]^2+</td>
<td>[M + 3H]^3+</td>
<td>Relative intensity normalized to 10(^{6})</td>
</tr>
<tr>
<td>G0F</td>
<td>1317.527</td>
<td>878.887</td>
<td>23.60</td>
</tr>
<tr>
<td>G1F</td>
<td>1396.553</td>
<td>932.705</td>
<td>28.06</td>
</tr>
<tr>
<td>G2F</td>
<td>1479.58</td>
<td>996.722</td>
<td>17.31</td>
</tr>
<tr>
<td>G6FN</td>
<td>1416.667</td>
<td>946.38</td>
<td>5.17</td>
</tr>
<tr>
<td>G1FN</td>
<td>1500.063</td>
<td>1000.398</td>
<td>7.41</td>
</tr>
<tr>
<td>G2FN</td>
<td>1591.119</td>
<td>1054.416</td>
<td>1.50</td>
</tr>
<tr>
<td>G1FS</td>
<td>1544.101</td>
<td>1029.737</td>
<td>2.82</td>
</tr>
<tr>
<td>G2FS</td>
<td>1625.127</td>
<td>1083.754</td>
<td>11.62</td>
</tr>
<tr>
<td>G1FNS</td>
<td>1645.641</td>
<td>1097.43</td>
<td>0.39</td>
</tr>
<tr>
<td>G2FNS</td>
<td>1726.687</td>
<td>1151.447</td>
<td>0.39</td>
</tr>
<tr>
<td>G0</td>
<td>1244.498</td>
<td>830.001</td>
<td>0.61</td>
</tr>
<tr>
<td>G1(^4)</td>
<td>1235.524</td>
<td>884.019</td>
<td>–</td>
</tr>
<tr>
<td>G2(^4)</td>
<td>1406.551</td>
<td>938.036</td>
<td>–</td>
</tr>
<tr>
<td>G0N</td>
<td>1346.036</td>
<td>899.694</td>
<td>0.19</td>
</tr>
<tr>
<td>G1N</td>
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<td>951.712</td>
<td>0.30</td>
</tr>
<tr>
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<td>1508.009</td>
<td>1056.73</td>
<td>0.30</td>
</tr>
<tr>
<td>G1S</td>
<td>1471.072</td>
<td>981.651</td>
<td>0.06</td>
</tr>
<tr>
<td>G2S</td>
<td>1552.089</td>
<td>1035.086</td>
<td>0.48</td>
</tr>
</tbody>
</table>

\(^a\) Calculated monoisotopic m/z values of the [M+2H]\(^2+\) and [M+3H]\(^3+\) species are given.  
\(^b\) Glycan compositions are given in terms of number of galactoses (G0, G1, G2) and presence of fucose (F), bisecting N-acetylgalactosamine (N), and sialic acid (S).  
\(^c\) The areas of the first, second and third isotopic peaks of the double protonated and triple protonated species were summed.  
\(^d\) Glycopeptides were excluded because of co-migrating contaminant in CE separation only present in the standard but not in patient samples.
The limits of detection and the dynamic range of the t-ITP-CZE-MS method were determined as described previously [6]. Briefly, 250 nl of two monoclonal antibody tryptic digest dilutions at 4, 16, 63, 250, 1000 and 4000 pg/µl were analyzed by a single analysis per concentration. In the Fc portion of each antibody two N-glycans are located, which may vary in glycan composition. Upon analysis of the lowest measurable concentration (16 pg/µl) three different glycoforms were observed in the analysis of both antibodies. The lower limit of detection (LOD) was estimated to be 20 amol for the individual glycopeptides which amounts to 80 pM sample concentration and was calculated by:

\[ \text{LOD} = \frac{\text{Lowest measurable antibody concentration (16 pg/µl)} \times \text{Number of Fc N-glycans (2)}}{\text{Molecular weight antibody (150 000)} \times \text{Number of observed major glycoforms (3)}} \]

This is an improvement in sensitivity by a factor of 40 in the lower LOD for t-ITP-CZE-MS as compared to nano-RPLC-MS. The higher LOD (detector saturation) was not “challenged” as the high concentration samples are always analyzed by the RPLC-MS method [6]. Nevertheless, the detection was observed to be linear over at least 2 orders of magnitude from the low LOD with R2 for both dilutions series above 0.99 for the three observed glycans. Notably, the achieved sensitivity is also better than that of recently tested MALDI-TOF-MS methods which achieved an LOD of between 40 and 400 amol on the MALDI target spot, depending on the applied matrix, sample preparation method, and ionization mode [6]. With regard to the MALDI-TOF-MS methods, it has to be mentioned that the LOD was determined using dilution series of highly concentrated and pure IgG, thereby limiting the potential negative influence of salts on the ionization process. One has to take into account, however, that the reported sensitivity will most probably not be achieved on scarce biological samples, as those will require additional sample preparation/desalting steps with the concomitant risk of sample loss.

The t-ITP-CZE system was then applied to analyze the IgG Fc-glycosylation of anti-Human Platelet Antigen 1a (HPA1a) allo-antibodies purified from serum of pregnant women. Briefly, allo-antibodies were affinity-purified exploiting their binding to the HPA1a antigen and subsequently digested with trypsin [5]. After digestion samples were dried down and reconstituted in 2.5 µl of 100 mM ammonium acetate buffer which functions as a leading electrolyte. Analysis of 250 nl aliquots by t-ITP-CZE provided high-quality IgG1 Fc glycopeptide profiles. These IgG1 Fc glycopeptide profiles reveal vast differences in core-fucosylation and galactosylation as illustrated in Figure 2-2. Sample 1 showed a high degree of both core-fucosylation and galactosylation (integration of the data revealed...
that 81% of glycans were fucosylated and 81% of the possible sites were galactosylated; Figure 2-2A) while sample 2 showed a very low degree of core-fucosylation and high degree of galactosylation (only 17% of all glycans were fucosylated while galactosylation was 83%; Figure 2-2B). Finally, sample 3 exhibited a high degree of core-fucosylation but low galactosylation (86% and 48% respectively; Figure 2-2C). This is consistent with previous observations by nano-RPLC-MS where we found heterogeneity of anti-HPA IgG1 allo-antibodies with regard to the degree of core-fucosylation and galactosylation [5]. As mentioned above, these differences in core-fucosylation and galactosylation may be functionally important as low core-fucosylation can vastly enhance the pathogenicity of allo-antibodies via increased ADCC activity and low galactosylation may be indicative of a pro-inflammatory response [5, 11, 12].

4 Conclusion

In this paper we present a t-ITP-CZE-MS method as an auxiliary method to our nano-RPLC-MS glycopeptides profiling workflow. The obtained glycosylation profiles are very similar to those determined using the recently described nano-RPLC-MS method. As the sample treatment to change from nano-RPLC-MS to t-ITP-CZE-MS is performed in a very straightforward manner by drying the sample followed by reconstitution in the required sample buffer, the two analytical strategies are complementary to each other. Therefore, sample that contains glycopeptide concentrations below the limit of detection for the nano-RPLC-MS method can be re-analyzed using the high sensitivity t-ITP-CZE-MS method and provide additional data without any extensive sample preparation or use of additional untreated sample. Alternatively, the samples may be stored for several months at -20°C after evaporation, making it possible to analyze the samples at a later time point.

The developed t-ITP-CZE-MS method is of limited value for the analysis of large sample sets due to the rather low throughput (60 min per sample) as compared to the previously developed nano-RPLC-MS method (16 min per sample). However, t-ITP-CZE-MS will serve as a complementary technique to analyze those samples with IgG levels below the current detection limits of the nano-RPLC-MS method.
Figure 2-2: IgG Fc-glycosylation profiles of HPA1a alloantibodies purified from sera of three pregnant women. Extracted ion traces are given of the major core-fucosylated (left) and non-core-fucosylated (right) glycopeptide species. The first HPA1a glycosylation profile is high in fucosylation, galactosylation and sialylation (A), the second shows low fucosylation with high galactosylation and sialylation (B), and the third shows low galactosylation and sialylation with high fucosylation (C). Blue square, N-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, N-acetylneuraminic acid.
References


