Transferrin - Protein, CZE
Analysis of Transferrin using a SMIL coating on deltaDOT’s HPCE platform

Transferrin isoforms including CDT were successfully analysed and resolved using the Peregrine High Performance Capillary Electrophoresis system and deltaDOT’s proprietary LFII® technology, demonstrating its ability to produce high quality reproducible data in terms of peak mobility (<0.5 % RSD) and peak area (~ 2 % RSD).

Most previous electrophoretic approaches to analyse these were highly complex and suffered from reproducibility problems.

Transferrin is a blood plasma glycoprotein involved in iron ion transport. Carbohydrate Deficient Transferrin (CDT) is one of the conventional markers for chronic alcoholism.

www.deltadot.com

Ref - dDAP02
INTRODUCTION

Transferrin (Tf) is a major iron transporting protein in serum. It is a glycoform and exhibits microheterogeneity in its amino-acid, iron and carbohydrate content. Tf has two asparagine linked N-glycan chains which are composed of neutral sugars forming di-, tri- and tetra antennary structures terminating with negatively charged sialic acid residues. The sialic acid content varies giving rise to isoforms with up to eight sialic acid residues. The molecular masses of these isoforms vary between 75 and 79 kDa and their isoelectric points range from 5.2 to 5.9.

A normal profile is characterised by a distribution of sialoforms, with the pre-dominant form being tetrasialo-Transferrin. The di-, tri-, penta-, and hexasialo-forms are found at markedly lower levels. Variation in serum sialoform content can be correlated to different pathological states. An increase in the relative levels of the lower sialoforms (i.e. disialo-Transferrin and asialo-Transferrin) has been associated with alcohol abuse. Carbohydrate deficient Transferrin (CDT) is the collective name for this group of less sialylated Transferrin isoforms.

This report demonstrates the capability of the Peregrine HPCE instrument for the label free analysis of Tf sialoforms using capillary zone electrophoresis (CZE). Separation of native proteins using CZE is problematic due to protein adhesion to the inner surface of the capillary. This can lead to unstable currents, inefficient separations, poor reproducibility and sometimes a lack of analyte elution. Using the Successive Multiple Ionic Layer (SMIL) coating developed by Katayama et al. 1998 [1] protein absorption was reduced and the Transferrin isoforms were separated based on differences in their net charge.

MATERIALS AND METHODS

The separation of the Transferrin glycoforms was performed in a bare fused silica capillary of 62 cm effective length and 50 µm internal diameter. The SMIL capillary coating was generated using the protocol detailed by Katayama et al. except that the capillary was rinsed using a proprietary methodology.

Electrophoresis was performed in borate buffer with a proprietary additive. The buffer was passed through a 0.2 µm filter and degassed before use. The samples were injected at 5 kV for 25 seconds and electrophoresed at a voltage of 16 kV. The samples were diluted in run buffer to a concentration of 1 mg/ml with 5 % ferric chloride solution. The sample was incubated for 10 minutes at room temperature before analysis to allow time for iron saturation.

The samples were desialylated by digestion with neuraminidase from Vibrio cholerae (BioChemika). Each 1 mg/ml Tf sample was treated with a 1 U/ml enzyme solution in a proprietary buffer. The samples were incubated at 37 °C for 5 hours. Aliquots were removed every 1 hour to assess the progress of the reaction. The digest was stopped by raising the pH to 8. Unused samples were stored at 4 °C.

Between runs the capillary was flushed using a proprietary methodology after which an automated Ohm’s Law Plot was performed.
RESULTS & DISCUSSION

The rapid and efficient separation of the Transferrin glycoforms was performed using a deltaDOT Peregrine system and data was analysed using both deltaDOT’s Equiphase Vertexing Algorithm (EVA) and General Separation Transform (GST) algorithm. GST is a method of combining the data from the 512 pixels in a natural way which preserves the peak shape information of the electropherograms while at the same time maximising the signal-to-noise ratio. A 10-fold increase in signal-to-noise using GST as compared to single electropherograms is typically observed. In addition, the absorbance \( A \) can be read directly off the GST plot peak height \( V \) as a function of time \( t \) (\( A = 0.434 \times V(t) \)).

EVA is an advanced pattern-recognition tool which maximizes the system resolution, and converts quantitative information to a peak height rather than area. In EVA the electropherograms are first analyzed to find local peaks. These are used first to perform vertexing (determine the point of origin of the bands) and then to produce a signal output.

Thiourea was used to test the strength and stability of the electro-osmotic flow generated by the SMIL coating which proved to be extremely stable. The percentage RSD over 40 runs for peak migration time, peak area and peak height were 0.7, 2.1 and 2.1 percent respectively. The GST and EVA processed data for the Holo-Transferrin sample is shown in Figure 1.

Figure 1: Panels A and B show GST electropherogram and EVA processed data for Human Holo-Transferrin. Peak identification: P2 – disialo-Tf, P3 – trisialo-Tf, P4 – tetrasialo-Tf, P5 – pentasialo-Tf and P6 – hexasialo-Tf

<table>
<thead>
<tr>
<th></th>
<th>mean</th>
<th>std dev</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Time (in minutes)</td>
<td>15.35</td>
<td>0.057</td>
<td>0.37</td>
</tr>
<tr>
<td>Peak Area</td>
<td>0.0191</td>
<td>0.00039</td>
<td>2.04</td>
</tr>
<tr>
<td>Peak Height</td>
<td>0.000475</td>
<td>9.713E-06</td>
<td>2.04</td>
</tr>
</tbody>
</table>

Table 1: EVA reproducibility data for Peak migration time, peak area and peak height for tetra-sialoform Transferrin (Tf-4) \( (N=10) \)

<table>
<thead>
<tr>
<th></th>
<th>mean</th>
<th>std dev</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Time (in minutes)</td>
<td>15.54</td>
<td>0.059</td>
<td>0.38</td>
</tr>
<tr>
<td>Peak Area</td>
<td>0.0048</td>
<td>7.78E-05</td>
<td>1.6</td>
</tr>
<tr>
<td>Peak Height</td>
<td>0.00012</td>
<td>1.91E-06</td>
<td>1.58</td>
</tr>
</tbody>
</table>

Table 2: EVA reproducibility data for peak migration time, peak area and peak height for penta-sialoform Transferrin (Tf-5) \( (N=10) \)
The EVA processed data for the neuraminidase treated Transferrin samples are detailed below. The results are shown as EVA overlays between the hourly aliquots to demonstrate the progress of the reaction. The development of the reaction can be monitored by the appearance of desialylated isoforms and the decrease in peak area of the higher sialoforms. At one hour (Figure 2) the major sialoform, Tetrasialo Tf shows a marked decrease along with the pentasialo Tf and an increase in tri- and di-sialo Transferrin and the appearance of the mono sialylated isoform. As the reaction developed the data showed the progressive decrease in all the higher sialoforms. After 5 hours (Figure 5) the major peaks present were the asialylated and monosialylated Transferrin isoforms.

The shift in peaks from the 1 hour aliquot to the 5 hour aliquot is made obvious by the EVA data overlay (Figure 6). The stability and reproducibility of the coating and the HPCE instrument allows the chronological monitoring of the enzyme-based desialylation of Transferrin.

Figure 2: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 1 hour (black) and 2 hours (blue). Peak identification: P1-monosialo-Tf, P2-disialo-Tf, P3-trisialo-Tf, P4-tetrasialo-Tf and P5-pentasialo-Tf

Figure 3: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 2 hours (black) and 3 hours (blue). Peak identification: P0-asialo-Tf, P1-monosialo-Tf, P2-disialo-Tf, P3-trisialo-Tf and P4-tetrasialo-Tf.

Figure 4: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 3 hours (black) and 4 hours (blue). Peak identification: P0-asialo-Tf, P1-monosialo-Tf, P2-disialo-Tf, P3-trisialo-Tf and P4-tetrasialo-Tf.

Figure 5: Overlay of EVA processed data of Human Holo-Transferrin after treatment with neuraminidase for 4 hours (black) and 5 hours (blue). Peak identification: P0-asialo-Tf, P1-monosialo-Tf, P2-disialo-Tf and P3-trisialo-Tf.
CONCLUSION
There are several electrophoretic approaches that have been shown to be effective in the resolution of Transferrin isoforms, however most are difficult to set up and do not provide reproducible results. The analysis of Transferrin isoforms on the deltaDOT HPCE platform provides a new, reliable tool for the analysis of CDT isoforms with the aid of the SMIL coating.

The advantages offered by the deltaDOT HPCE instrument in the analysis of Transferrin are simplicity of operation, speed of separation and the resolution obtained between the sialoforms. The robustness of the technique is demonstrated by the stability of the coating and the reproducibility of the results obtained.

REFERENCE