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Application Note

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CIM® @Tf-0.2 Monolithic 96-Well Plate for Immunoaffinity Isolation of Transferrin From Human Plasma

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Abstract

Transferrin (Tf) is an iron-binding glycoprotein found in blood plasma. The protein is of clinical importance because its glycosylation patterns are a marker for some disease states in humans. Robust, fast, and cost-effective methods for reliably purifying the protein at high throughput are essential to support improved clinical understanding of how Tf functions in healthy and diseased states.

This application note describes the development of an immunoaffinity purification method on a monolith column with immobilized anti-transferrin antibodies and the successful transfer of the method to the monolithic 96-well plates.

Introduction

Convective Interaction Media (CIM®) monolithic supports are an ideal tool for the high-throughput separation of large biomolecules (such as proteins, viruses, and plasmid DNA) due to their large, highly interconnected channels, high dynamic binding capacity for large molecules, and very low backpressure. Monolithic plates with immobilized Protein A or Protein G have previously been used for the high throughput isolation of immunoglobulins from complex biological samples—like human plasma—and are reusable and stable over long periods.

Transferrin (Tf) is a glycoprotein that transports iron to cells and has two N-glycosylation sites—at asparagine 432 and asparagine 630—in humans. Carbohydrate-deficient Tf, which lacks one or both N-glycans, is the most common marker for congenital disorders of glycosylation.[1] Altered Tf glycosylation has also been reported in hepatocellular carcinoma[2] and chronic alcohol consumption.[3,4] High-throughput Tf purification and glycan characterization methods are under extensive development to facilitate glycosylation pattern screening for population, genetic, and clinical studies.

This application note describes the development of an immunoaffinity purification method on a CIMac™ analytical column with immobilized anti-transferrin antibodies (@Tf) and the successful transfer of the method to the monolithic 96-well plate (CIM® @Tf-0.2 monolithic 96-well plate). The affinity purification method was used to isolate Tf from human blood plasma, followed by ultra-performance liquid chromatography (UPLC) analysis of Tf N-glycosylation.

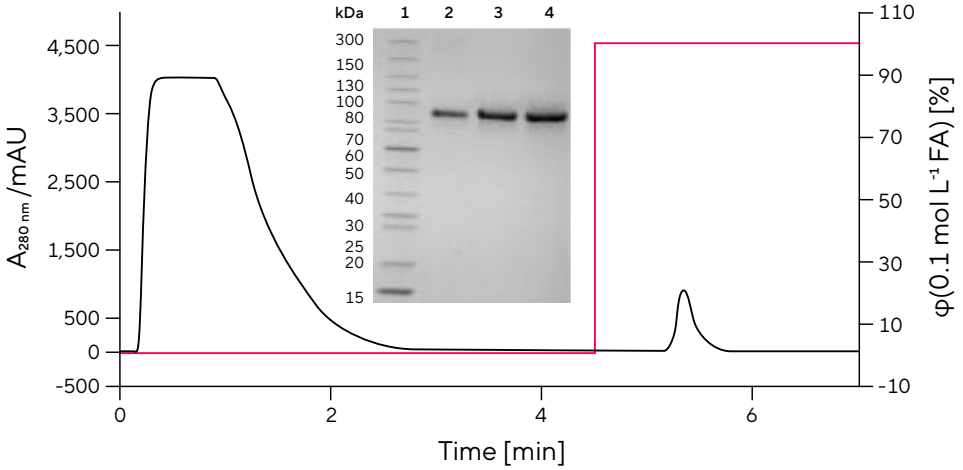
Methods and Results

Mouse-derived monoclonal @Tf antibodies specific for human Tf were produced at the Faculty of Medicine, University of Rijeka, Rijeka, Croatia. They were covalently attached to the hydrazide-derived surface of chromatographic monoliths to achieve favorable orientation of the antibody Fab region into the lumen of monolithic channels [ref]. This method achieved a dynamic binding capacity of 3.7 mg of human Tf per mL chromatographic support. Optimization of the Tf isolation | purification from human plasma was done on CIMac™ @Tf column, shown in Figure 1, and the immunofinity purification method is shown in Table 1.

Table 1: *Immunoaffinity Purification Method*

Process Item	Details
Column	▪ CIMac™ @Tf-0.1 analytical column OR ▪ CIM® @Tf-0.2 monolithic 96-well plate
Plate conditioning	▪ 10 CV ultra-pure water (18 MΩ cm at 25 °C) ▪ 5 CV 0.1 M formic acid, pH 3.0 (pH adjusted with NH_3, aq) ▪ 20 CV phosphate-buffered saline (PBS) pH 7.4
Sample load	100 µL of plasma | serum sample, 7× diluted with 1× PBS, pH 7.4, and depleted of IgG [ref]
Washing	3 times 10 CV 1× PBS, 0.25 M NaCl, pH 7.4
IgG elution	3.5 CV 0.1 M formic acid, pH 3.0 (pH adjusted with NH_3, aq)
IgG neutralization	1 M ammonium hydrogen carbonate to pH 7.0
Plate regeneration	▪ 10 CV 0.1 M formic acid, pH 3.0 (pH adjusted with NH_3, aq) ▪ 20 CV 1x PBS, pH 7.4
Storage	1× PBS +0.02% NaN3, pH 7.4; keep at 4 °C

Figure 1: *Tf Isolation | Purification From Human Plasma on the CIMac™ @Tf Column*



Note. Yellow line—typical HPLC-UV profile of plasma loading and elution from CIMac™ @Tf column.
Teal line—elution of bound proteins with 0.1 mol L^{-1} formic acid. Insert—SDS-PAGE gel of Tf standard and Tf isolated from human plasma in 1st and 4th consecutive isolations on the same column.

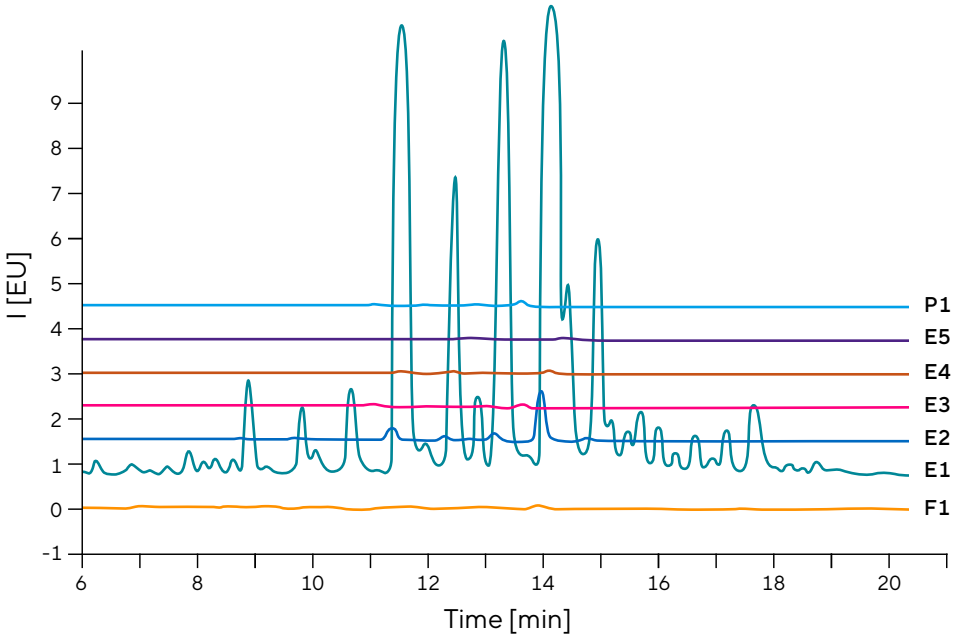
Lanes:
1. 10–200 kDa molecular mass standard (Fermentas Life Sciences, Burlington, Canada)
2. Standard human Tf (0.1 mg mL^{-1})
3. Tf elution from the 1st isolation
4. Tf elution from the 4th isolation.

Around 60 µg of Tf eluate was dried and deglycosylated. Free glycans were labeled with 2-aminobenzamide and, after clean-up, analyzed by hydrophilic interaction liquid chromatography-ultra performance liquid chromatography (HILIC-UPLC) using Waters™ ACQUITY™ UPLC™ Glycan BEH™ Amide column (Figure 2, Figure 3).

The method was successfully transferred to the 96-well format. The Tf elution capacity was within previously determined requirements for the amount of purified Tf, and the newly prepared CIM® @Tf-0.2 monolithic 96-well plate could be used for larger population studies. Figure 4 shows the representative HILIC-UPLC chromatogram of Tf N-glycan peaks from a single well isolation on a 96-well plate.

Figure 2: *Schematic of Sample Processing and Analysis*

Figure 3: *Glycosylation Profiles of Fractions Collected During Tf Isolation from Human Blood Plasma on CIMac™ @Tf Column*



Note.

P1 Wash with 1 mL of 1× PBS, pH 7.4. EU (emission units)[5]

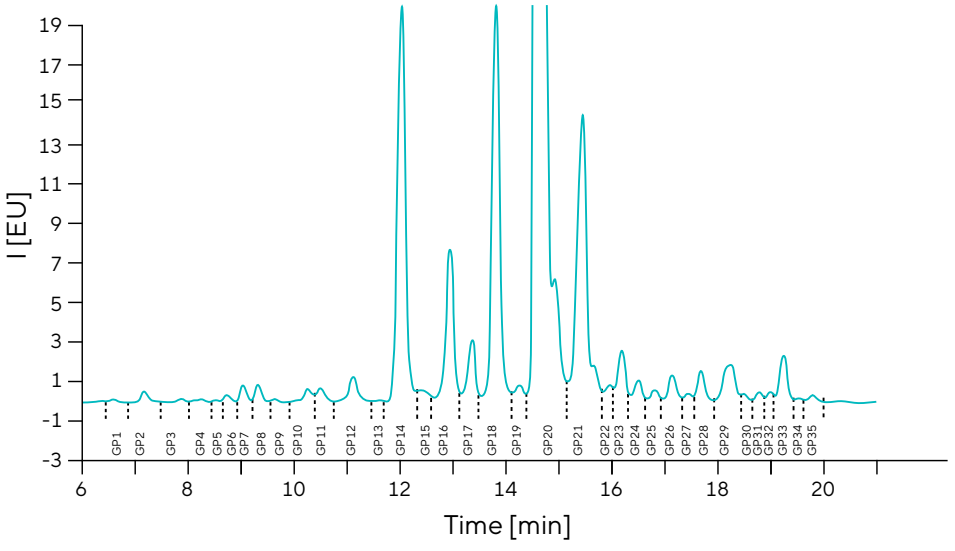
E1, E2 Tf elution fractions with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 (0.5 mL each

E3 Additional wash with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0

E4, E5 Additional wash with 1 mL of 0.1 mol L^{-1} formic acid, pH 2.5 (0.5 mL each)

F1 Wash with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 (pH adjusted with ammonia solution, φ = 25%) before plasma sample application

Figure 4: *Representative HILIC-UPLC chromatogram of Tf N-Glycan Peaks (GP1-GP35)*



Note. EU = emission units

Conclusion

CIM® @Tf-0.2 monolithic 96-well plates enable fast, reproducible, and specific isolation of human Tf from blood plasma. This technology represents an essential tool for high-throughput Tf glycosylation analysis workflow. CIM®-@ Tf-0.2 monolithic 96-well plate and CIMac™ @Tf-0.1 Analytical Columns are stable for over a year and have been re-used 20 times for Tf isolation from human plasma without significant loss of binding capacity.

CIM® monolithic 96-well plates could be equally successfully coupled with other specific monoclonal or polyclonal antibodies to make them applicable for specific isolations of different proteins or other molecules from biological samples.

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