Seok-Joon Kwon[1]
Fuming Zhang[1]
Jonathan S. Dordick[1,2,3]
William J. Sonstein[4]
Robert J. Linhardt[1,3,4,5]

[1]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA
[2]Department of Biology, Rensselaer Polytechnic Institute, Troy, NY, USA
[3]Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA
[4]Neurological Surgery, P.C., Rockville Centre, NY, USA
[5]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY, USA

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Research Article

Detection of cerebrospinal fluid leakage by specific measurement of transferrin glycoforms

A simple and rapid detection of cerebrospinal fluid (CSF) leakage would benefit spine surgeons making critical postoperative decisions on patient care. We have assessed novel approaches to selectively determine CSF β2-transferrin (β2TF), an asialo-transferrin (aTF) biomarker, without interference from serum sialo-transferrin (sTF) in test samples. First, we performed mild periodate oxidation to selectively generate aldehyde groups in sTF for capture with magnetic hydrazide microparticles, and selective removal with a magnetic separator. Using this protocol sTF was selectively removed from mixtures of CSF and serum containing CSF aTF (β2TF) and serum sTF, respectively. Second, a two-step enzymatic method was developed with neuraminidase and galactose oxidase for generating aldehyde groups in sTF present in CSF and serum mixtures for magnetic hydrazide microparticle capture. After selectively removing sTF from mixtures of CSF and serum, ELISA could detect significant TF signal only in CSF, while the TF signal in serum was negligible. The new approach for selective removal of only sTF in test samples will be promising for the required intervention by a spine surgeon.

Keywords:
Cerebrospinal fluid / Glycoforms / Transferrin DOI 10.1002/elps.201500128

1 Introduction

Spinal fluid leak as a result of incidental durotomy during spinal surgery is a relatively common complication that occurs with an incidence of 2–17% [1–6]. Most of time, spinal fluid leaks are recognized at the time of surgery and successfully repaired. Occasionally they present in a delayed fashion if a small durotomy is not recognized at the time of surgery or if the repair is not ideal initially. Spine surgeons are frequently confronted with postoperative fluid collections that may or may not represent a CSF (cerebral spinal fluid) leak. This is more commonly an issue with lumbar spine surgery for degenerative disease. If a patient presents with positional headaches or with clear fluid leakage, then the diagnosis is more easily made. However, in the postoperative period it is sometimes confounding differentiating seromatous fluid from CSF as a patients' symptoms do not always classically present. A patient may present with a bulging subcutaneous collection of fluid whereupon aspiration, the nature of the fluid is not certain. In surgical decision-making, it would be ideal to confirm the diagnosis of CSF leak quickly so that one can initiate repair, which requires surgical intervention particularly if there is skin drainage, which could result in meningitis. It would be advantageous to know if the collection is a seroma as these can often be treated conservatively without return to the operating room. Currently to distinguish CSF from seromatous fluid, one must send out the fluid sample to a laboratory utilizing electrophoresis, which could take 3–5 days for the results.

A combination of protein separation and detection, using electrophoresis and MS, has been successfully applied to identify protein biomarkers in CSF [7]. Transferrin (TF) isoforms among protein biomarkers in CSF have been used as a critical diagnostic marker not only for detecting CSF leakage from liquorrhea but also detecting several diseases, including early stage oral cancer [8], chronic alcoholism [9], and diabetic kidney disease [10].

TF is a secreted glycoprotein, having multiple glycoforms, containing glycans capped at their nonreducing ends with negatively charged sialic acid residues [11, 12]. TF plays a crucial role in homeostasis and transport of iron, as well as in protecting the body against free radical damage associated with unbound iron [13]. TF in serum is composed of 679 amino acid residues (~78 kDa MW) and has two glycosylation sites at asparagine Asn432 and Asn630 that are often occupied by N-linked glycans harboring various number of terminal (nonreducing end) sialic acid (or N-acetylneuraminic acid) residues, resulting in a heterogeneous populations of TF glycoforms [11, 14] (Fig. 1). TF in serum is exclusively

Correspondence: Professor Robert J. Linhardt, Rensselaer Polytechnic Institute, Biotech 4005, 110 8th St., Troy, NY 12180, USA
E-mail: linhar@rpi.edu
Fax: +1-276-3405

Abbreviations: **β2TF**, β2-transferrin; **aTF**, asialo-transferrin; **CSF**, cerebrospinal fluid; **sTF**, sialo-transferrin

Colour Online: See the article online to view Figs. 1-5 in colour.

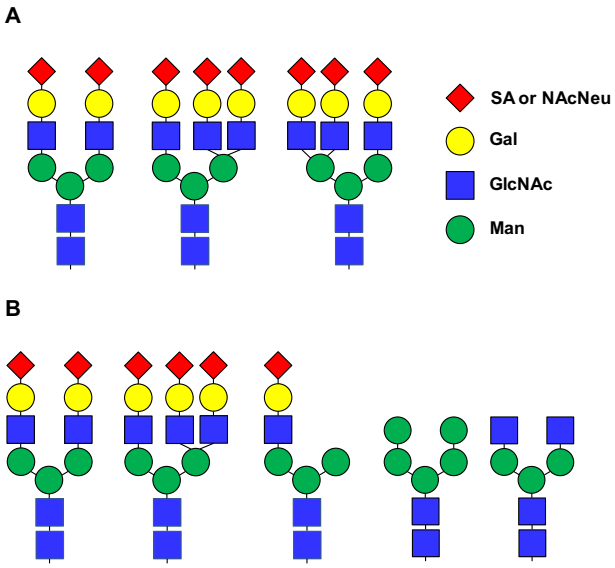


Figure 1. Structure of glycans present in transferrin glycoforms. (A) Serum sTF; (B), CSF sTF, and aTF, β2TF.

comprised of fully sialylated glycoforms. In contrast, TF in CSF, referred to as β2-transferrin (β2TF), exists as a mixture of sialo (sTF), and asialoglycoforms (aTF) [7, 12] (Fig. 1). It has been speculated that the aTF in CSF originates from serum sTF through the action of brain neuraminidase [15]. Over the years, a number of methods have been designed to detect TF isoforms as biomarkers of CSF leakage, as well as various disorders of the central nervous system [16, 17]. Different separation methods relying on electrophoresis have been developed to separate TF isoforms, including isoelectric focusing [18], immunofixation gel electrophoresis [19], SDS-PAGE [20], and CE [21].

Herein, we develop a simple and rapid method enabling a spine surgeon to detect CSF directly from postoperative drainage. Spine surgeons can be faced with critical, time sensitive decisions regarding patient care when a fluid leak is detected at the surgical site of a patient's postoperative incision. The current test for identifying CSF from the leak generally takes 3–5 days to obtain the results from an independent clinical laboratory, which can be too long to effectively treat patients. To these ends, we have developed novel chemical and enzymatic methods to specifically oxidize serum sTF allowing it to be conjugated to hydrazide magnetic microparticles and selectively removed from a test sample, allowing the rapid detection of CSF-derived aTF by a method amenable to use in rapid, real-time "dip-stick" analysis.

2 Materials and methods

2.1 Enzyme reactions and labeling

Human serum sTF (10 mg/mL, Sigma) was dissolved in 1X Glycobuffer (20 mM sodium acetate buffer at pH 5.5, including 5 mM $CaCl_2$). The human sTF solution was treated with neuraminidase (1 mg/mL in 1X Glycobuffer, Sigma) at 37°C overnight to generate aTF. Both sTF and aTF were labeled with NHS-rhodamine (Pierce) following the manufacturer's protocol. All unreacted rhodamine was removed by column chromatography on a PD MiniTrap G-25 column (GE Heathcare).

A two-step enzymatic reaction including neuraminidase (Sigma) and galactose oxidase (Sigma) was performed on human CSF (PrecisionMed), on pooled human serum (Innovative Research Inc.) and on individual human serum samples (BioreclaimationIVT). Both CSF and serum were first diluted twofold and 200-fold, respectively, in 1X Glycobuffer. Diluted CSF (10 μL) and diluted serum (10 μL) were each treated for 1 h with neuraminidase (10 μL of 1 mg/mL) at 37°C, followed by treatment for varying times with galactose oxidase, dissolved in 100 mM, pH 7.2, Tris buffer (10 μL of 0.5 KU/mL) at 37°C.

2.2 Agarose gel electrophoresis

After dissolving 0.4 g of agarose powder (Sigma) in 40 mL of 1X Tris-borate buffer (89 mM Tris base and 89 mM boric acid, pH 8.0), agarose gel (1%) was prepared by melting the agarose in a microwave oven and then the melted agarose solution was poured into the casting tray, forming a solid gel after cooling at room temperature. Loading samples were prepared by mixing the rhodamine-labeled proteins (20–320 ng in 10 μL) with 30% glycerol (2 μL). After loading the protein samples, the gel was subjected to electrophoresis at 200 V for 15 min.

2.3 Periodate oxidation

Mild periodate oxidation was performed with 1 mM $NaIO_4$ at 4°C (on ice) for 30 min, to oxidize the nonreducing end sialic acid residues in TF. Excess periodate and formaldehyde, generated during periodate oxidation, were removed by PD Mini Trap G-25 column (GE Heathcare). After desalting and buffer exchange with 100 mM, pH 7.0, sodium phosphate buffer using a G-25 column, the TF containing oxidized sialic acid residues were captured with SiMAG-Hydrazide microparticles (Chemicell).

2.4 Coupling and separation of sialo-proteins

After washing SiMAG-Hydrazide particles (10 mg/mL) twotimes with pH 7.0, 100 mM sodium phosphate buffer, the particles were incubated with TF containing oxidized sialic acid residues for 3 h at 20°C. The protein–particle conjugates were pelleted using a magnetic separator. Proteins remaining in the supernatant were collected and concentrated with Amicon ultracentrifugal filters (Ultracel-3K). The concentrated samples containing aTF were analyzed by agarose gel electrophoresis or using a transferrin ELISA kit (Abcam).

2.5 ELISA assay

Human TF in test samples could be detected with transferrin ELISA assay kit (Abcam). Briefly, standards or test samples were added to the 96-well plates precoated with TF specific antibody, then specific biotinylated TF detection antibody was added, and the plates were washed with wash buffer. Streptavidin-peroxidase complex was added and unbound conjugates were washed away with wash buffer. TMB was used to visualize streptavidin-peroxidase enzymatic reaction as TMB is catalyzed by peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The absorbance of yellow color was immediately measured with a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 450 nm. The detailed ELISA protocols were followed by manufacture's guideline (Abcam).

3 Results and discussion

Neuraminidase was used to generate aTF from sTF. After removal of sialic acid residues, TF was labeled using NHS-rhodamine (Fig. 2A). Rhodamine-labeled aTF and sTF could be separated using agarose gel electrophoresis (Fig. 2B). The results showed that more negatively charged sTF migrated closer to the anode. In addition, rhodamine-labeled transferrin could selectively detected in human plasma (Fig. 2B). The detection limit for rhodamine-labeled TF was 2 μg/mL (Fig. 2C), which is similar to detection by immunofixation gel electrophoresis [19].

Because human serum TF is a glycoprotein with two glycans containing nonreducing terminal sialic acid residues (sTF) and CSF contains both sTF and aTF [7, 12], mild periodate oxidation [22] renders sTF capturable as a hydrazone (Fig. 3A). Thus, we selectively removed sTF to facilitate the detection of aTF in CSF (Fig. 3A). The terminal sialic acid residues in sTF were oxidized to their aldehyde derivatives by mild treatment with sodium periodate [22, 23], and the oxidized sTF was captured by its covalent coupling to SiMAG-Hydrazide (magnetic hydrazide microparticles) in the form of a stable hydrazone linkage. The sTF-beads conjugate could then be easily removed using a magnetic separator (Fig. 3B). As a proof of concept, we subjected different concentrations (100 μL) of sTF and aTF to mild periodate oxidation (1 mM $NaIO_4$ at 4°C for 30 min), selectively introducing an aldehyde at the C-7 position of the terminal sialic acid residues in sTF. After removing unreacted oxidation reagent by desalting on a Sephadex G-25 column, the aldehyde group-containing sTF was captured by incubating with 200 μL of SiMAG-Hydrazide (10 mg/mL) at 20°C for 3 h. The captured sTF was removed with a magnetic separator and the supernatant, containing aTF with trace amounts residual sTF was assayed using agarose gel electerophoresis (Fig. 3C). The results showed that sTF was selectively removed through covalent capture with SiMAG-Hydrazide and that the aTF remained in supernatant buffer. At high concentrations of sTF (0.8 mg/mL), ~50% of sTF remained in supernatant buffer because of the presence of insufficient amounts of SiMAG-Hydrazide, required for its capture. When we doubled the amount of SiMAG-Hydrazide (20 mg/mL), the residual sTF remaining dropped to <3% (data not shown). This mild periodate oxidation quickly and selectively introduces aldehyde groups into sTF within 30 min (Fig. 3C) and coupling with SiMAG-Hydrazide magnetic microparticles can be accomplished 3 h requiring an overall pretreatment time of <5 h. Encouraged by these results, we applied the protocol developed to detect CSF aTF in samples consisting of mixtures of CSF and serum. Mixtures (10 μL) of rhodamine-labeled (200-fold) diluted serum and rhodamine-labeled (twofold) diluted CSF were subjected to mild periodate oxidation as a proof-of-concept test on a sample containing both CSF and serum. After selective separation of sTF from both serum and CSF following the protocol described above, residual sTF in agarose gel was analyzed (Fig. 4A). The level of sTF in serum was higher than the sTF in CSF and all the sTF present in both serum and CSF was successfully removed (bottom bands in Fig. 4A) by mild periodate oxidation and capture with SiMAG-Hydrazide.

It was not possible to detect directly aTF bands in agarose gel because of the complex mixture of proteins present in both serum and CSF. Human transferrin ELISA, therefore, was performed to specifically capture only TF in order to detect the residual TF in both CSF and serum (Fig. 4B). The amount of residual TF (aTF) in CSF was clearly higher than that of the residual TF (uncaptured sTF) in serum. However, trace amount of residual TF (uncaptured sTF) was still present in serum either because of incomplete oxidation of sialic acid residues or inefficient capture. Based on standard curve of TF in ELISA (data not shown), the level of residual trace TF in serum was ~7 ng/mL, similar to that of the buffer control. In contrast, residual TF (aTF) in CSF was ~60 ng/mL corresponding to one-thirds of the initial amount of TF in CSF (~170 ng/mL). This result was expected as only ~30% of the total TF in CSF is aTF [7]. We prepared mixtures of serum and CSF with different volume ratio to simulate real situation of CSF leakage. After selective removal of sTF from the mixtures of serum and CSF with the above protocols, residual TF (mainly aTF) was measured by ELISA kit (Fig. 4C). The results showed that the amounts of the residual TFs from the mixtures of serum and CSF were similar to those of the residual TF from different amounts of CSF in buffer, which is the same dilution of the mixtures of serum and CSF. In addition, we used multiple serum samples (S1: age 29/African male, S2: age 41/Caucasian male, S3: age 61/Hispanic female, S4: age 21/African female) and prepared the mixture (1:1 v/v ratio) of CSF and various serums and removed sTF selectively. Both the initial and residual TFs from the mixtures of different serum samples and CSF were measured by ELISA kit (Fig. 4D). The residual TF (mainly aTF) from CSF were clearly detected regardless of type of serum and the amounts of residual TF from serum were similar to negative control (only buffer).

Although we could discriminate between CSF and serum through the selective removal of sTF, the complete removal of

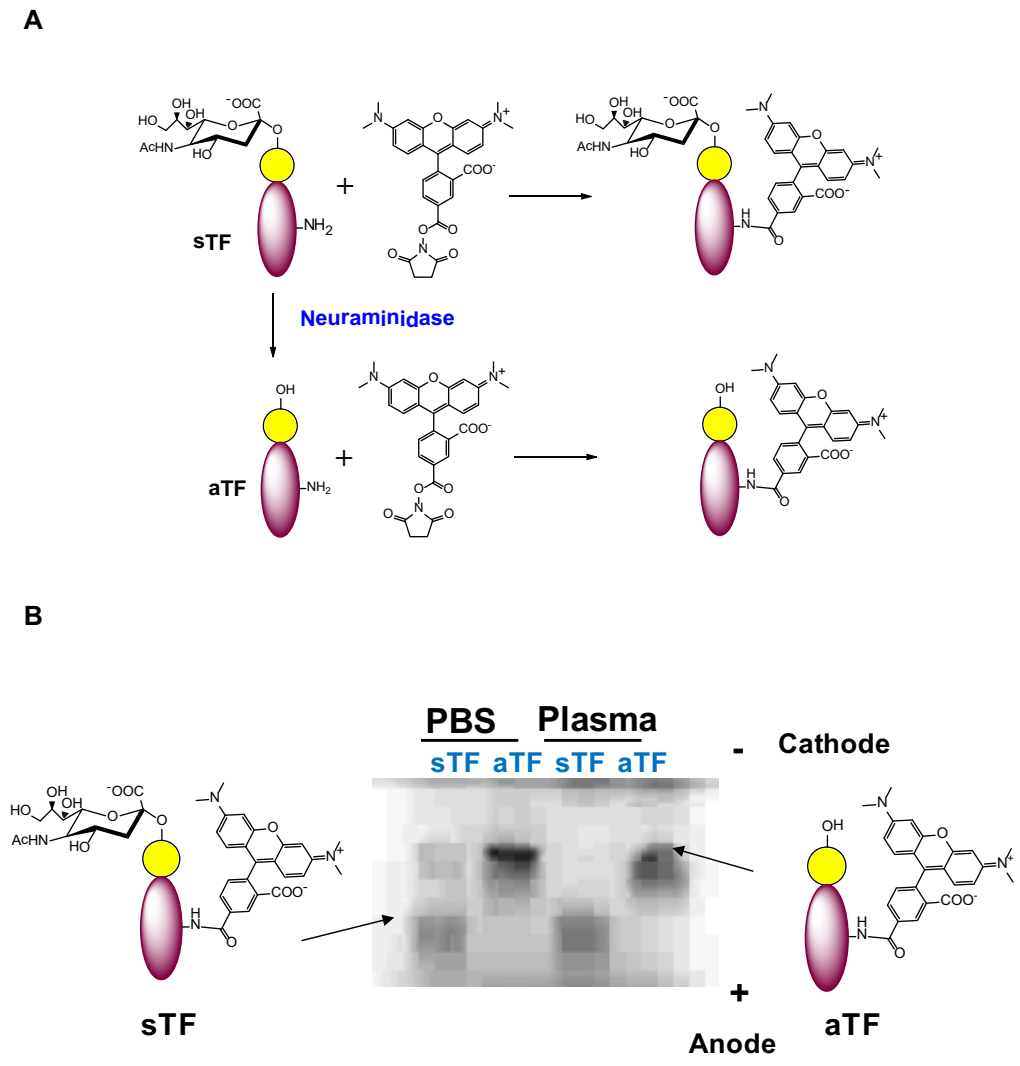
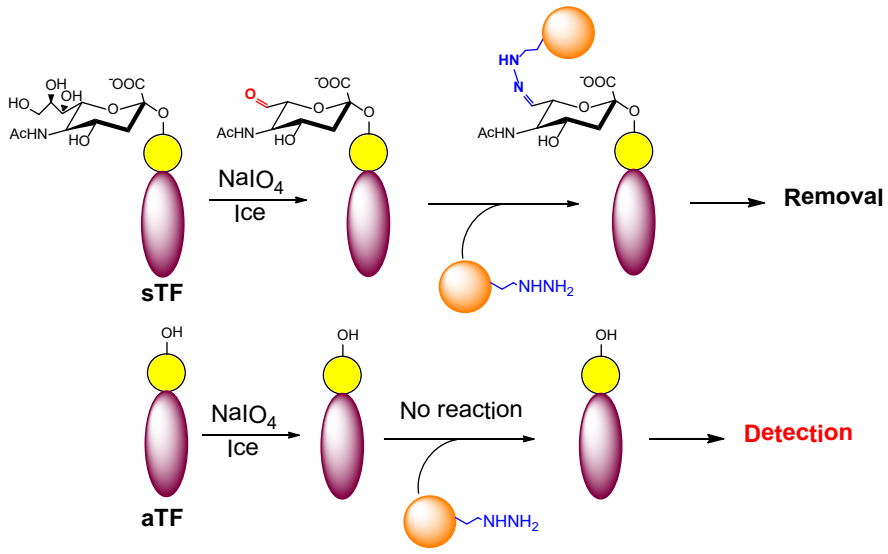
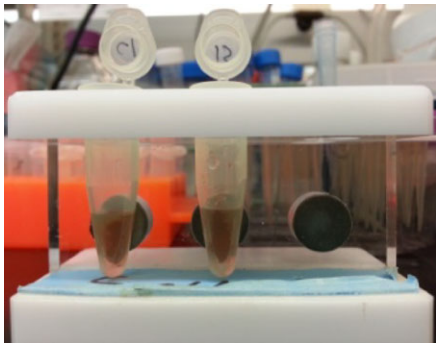


Figure 2. Electrophoresis-based assay for determination of TF glycoforms. (A) Fluorescent labeling of TF glycoforms sTF and aTF using NHS-rhodamine. (B) Separation of rhodamine-labeled aTF from rhodamine-labeled sTF. (C) Detection limit for rhodamine-labeled TF.

A



B



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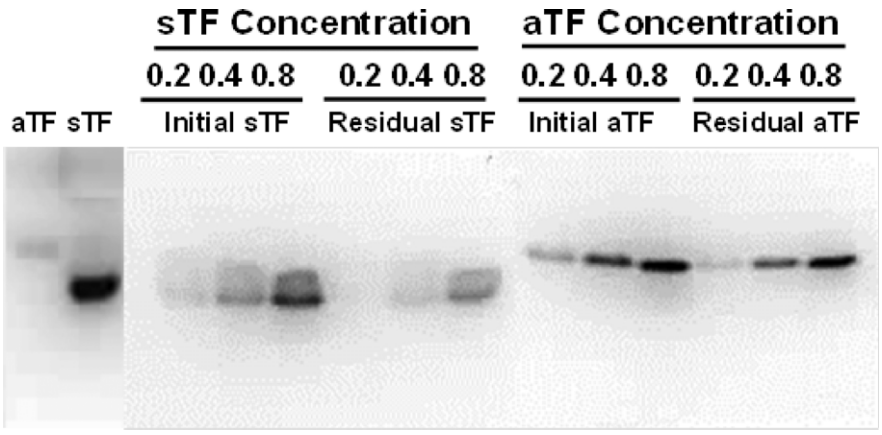


Figure 3. Single-step periodate glycan oxidation for the separation of TF glycoforms from buffer glycan. (A) Periodate oxidation of terminal sialic acid and capture with SiMAG-Hydrazide. (B) Magnetic separator to remove captured TF glycoform and recover supernatant. (C) Separation and detection of aTF and trace sTF in buffer by electrophoresis.

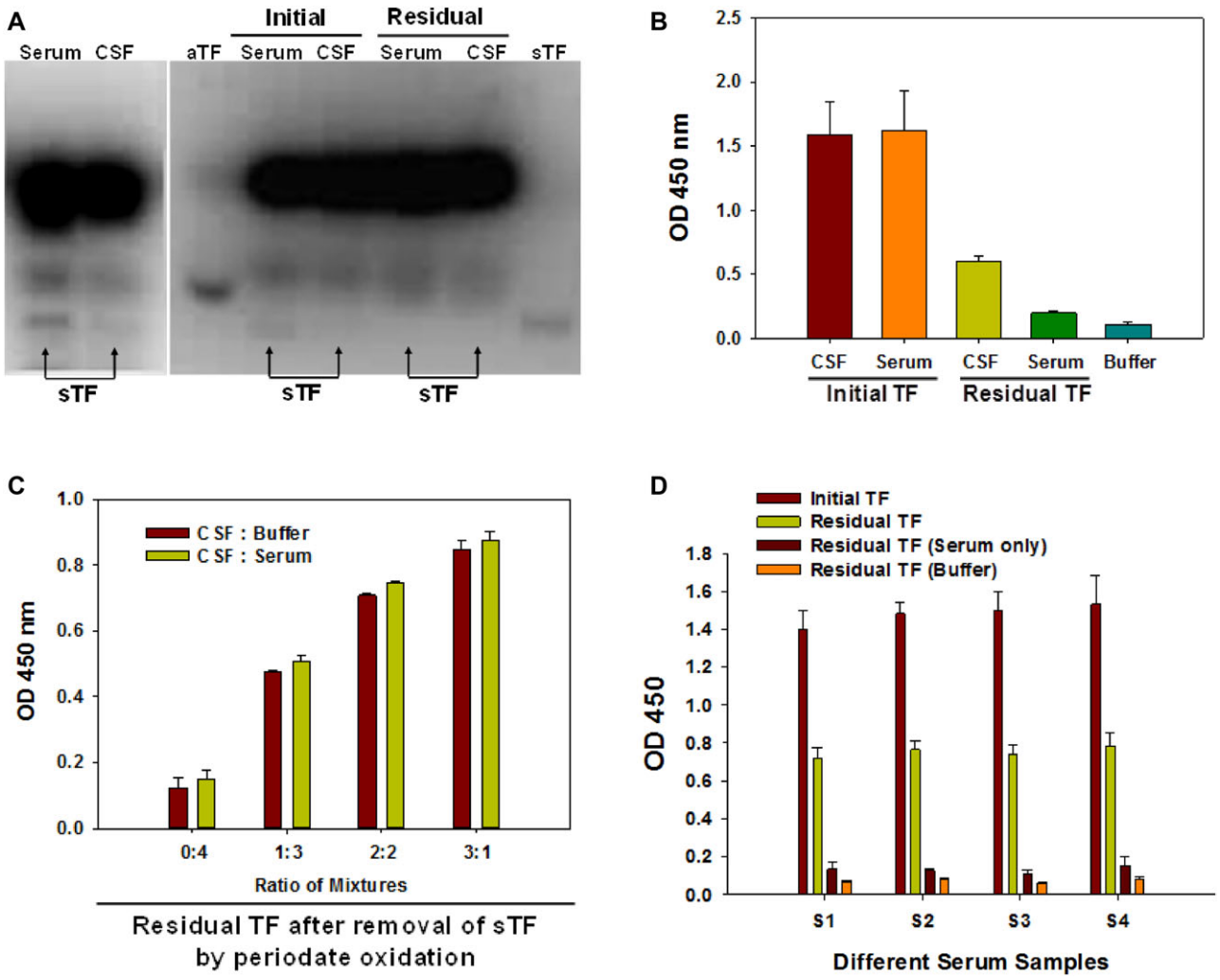
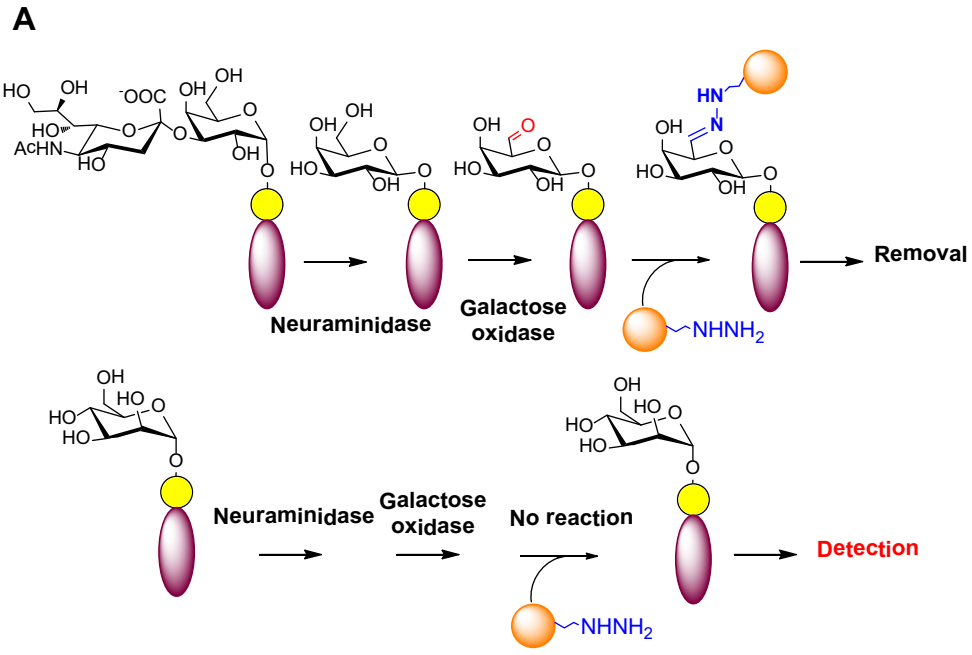


Figure 4. Separation of sTF from TF glycoforms using single-step periodate oxidation in mixture of CSF and serum. (A) Agarose gel separation and detection of sTF removed from both CSF and serum. (B) Quantification of TF in CSF and serum, respectively, before and after single-step periodate oxidation. (C) Quantification of residual TF (mainly aTF) in different mixtures of CSF and serum after single-step periodate oxidation. (D) Detection of TF after selective removal of sTF from mixture (1:1 v/v ratio) of CSF and various serums (S1: age 29/African male, S2: age 41/Caucasian male, S3: age 61/Hispanic female, S4: age 21/African female).

serum sTF is desirable for the accurate determination of aTF in CSF when analyzing mixtures of CSF and serum. Hence, we examined a two-step enzymatic (neuraminidase and galactose oxidase) reaction for generating aldehyde groups in sTF since enzymes show very high substrate specificity. Because there are neither nonreducing terminal sialic acid residues nor galactose residues in CSF-derived aTF [7], a two-step enzymatic reaction should be quantitative and selectively introduce aldehyde groups at the C-6 position of the galactose residue into sTF (Fig. 5A). A 10-μL sample of both diluted (twofold) CSF and (200-fold) serum was added to neuraminidase (10 μL of 1 mg/mL) and galactose oxidase (10 μL of 0.5 KU/mL) at 37°C. After subjecting the samples with two enzymes for different lengths of time, SiMAG-Hydrazide (100 μL of 10 mg/mL) was added to the reaction mixture and incubated for an additional 3 h at 20°C to capture the oxidized sTF. After pulling down the microparticle-captured oxidized sTF with a magnetic separator, residual TF in the supernatant was determined with ELISA (Fig. 5B). The results showed that we could completely remove serum sTF in this two-step enzymatic reaction, although this procedure required 24 h. However, although, this two-step process could successfully remove all the sTF, this pretreatment step required ~24 h, too long to accommodate a surgeon's immediate clinical decision. Consequently, future work will be aimed at improving the selectivity of periodate pretreatment, as well as decreasing the time required for the two-step enzymatic pretreatment.

Rapid and sensitive detection of CSF is crucial [24] to make real-time critical decisions regarding patient care. For example, if a CSF leakage occurs postsurgery, a patient may need to quickly return to the operating room to explore and repair the CSF leak, which would in turn treat the positional headaches and potential infection from contact with contaminated skin, thereby increasing the risk of developing



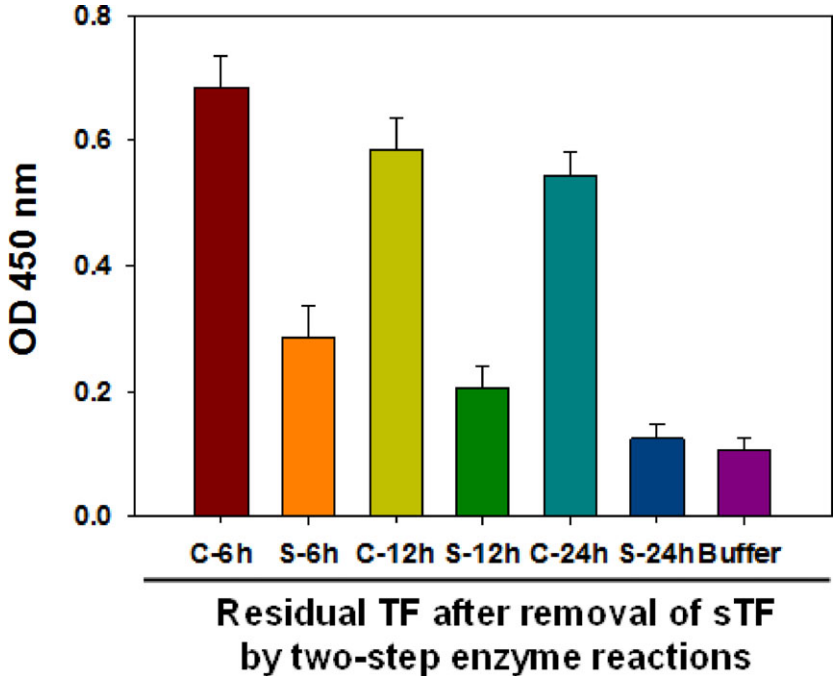


Figure 5. Two-step enzymatic oxidation for the separation of TF glycoforms. (A) Removal of sialic acid and oxidation of galactose followed by capture and removal of oxidized sTF. (B) Quantification of two-step enzymatic oxidation for the separation of TF glycoforms.

meningitis. At the time fluid is first noticed, and if the surgeon is unsure whether the fluid contains CSF, the surgeon can often only wait for confirmatory analysis, which delays action and can lead to poorer patient prognosis. In some cases a patient might not a classic presentation of a positional headache, which can further delay the diagnosis of a CSF fluid leak. Thus, a rapid test that can detect the presence of CSF fluid would allow spine surgeons to make immediate clinical decisions leading to improved patient outcomes.

4 Concluding remarks

The formation of β2TF is mediated by neuraminidase activity within the central nervous system [25]. Therefore, β2TF represent a potential highly selective marker protein for CSF leakage, since it is only located within the CSF where it is present as asialylated TF glycoforms, aTF. This anatomical selectivity enabled the development of a pretreatment method for rapid and selective removal of serum sTF in fluid leak samples, which would enable detection of CSF-associated β2TF (aTF). A rapid pretreatment method would also facilitate the commercial development of an easy to use a simple TF test kit.

The authors have declared no conflict of interest.

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