ASSESSING THE TRAUMATIC BRAIN INJURY MARKERS S100 AND C-REACTIVE PROTEIN IN HUMAN CEREBROSPINAL FLUID VIA MICROFLUIDIC IMMUNOSUBTRACTION

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ABSTRACT

Rapid and accurate assessment of protein mobility and binding specificity is achieved on-chip via a novel automated immunosubtraction assay developed on a re-useable microfluidic platform. The assay requires no chemical immobilization of proteins in the microchannel network, instead relying on a small pore size polyacrylamide sieving matrix adjacent a loading gel (12%T and 3%T respectively) which acts as a size-exclusion filter to subtract large target antigen-antibody complexes from subsequent electrophoresis. Results for detecting S100 (65 nM) in human cerebrospinal fluid indicate a lower limit of detection of ~3.25 nM, which is well within the clinically relevant range.

KEYWORDS: Immunosubtraction, Immunoassay, Electrophoresis, Traumatic Brain Injury, Polyacrylamide Gel, Cerebrospinal Fluid

INTRODUCTION

We report a microfluidic immunosubtraction assay for quantitation of putative traumatic brain injury (TBI) protein markers in raw human cerebrospinal fluid (CSF). To our knowledge, this work is the first microfluidic immunosubtraction assay reported. Immunosubtraction is a proven clinical assay capable of accurately quantifying target-analyte binding specificity and mobility in complex biological fluids. The use of immunosubtraction is gaining popularity in critical care scenarios. Consequently, we demonstrate microfluidic immunosubtraction for detecting clinically relevant levels of S100 and C-reactive protein (C-RP), putative biomarkers of TBI, in raw human CSF with assay durations of < 2 min. Our work has potential for use by first-responders to improve treatment and prognostic outcome for the 1.7 million people annually in the US alone who suffer brain injuries from sports, automobile accidents, domestic violence, and military combat [1].

EXPERIMENTAL

To yield both high-specificity binding and mobility information, on-chip immunosubtraction employs a homogeneous immunoassay format with filter-based exclusion (i.e., "subtraction") of large antibody-target complexes from subsequent electrophoresis (Figure 1). While powerful, current bench-top immunosubtraction is slow, labor intensive, requires qualitative electropherogram interpretation [2], and is available primarily for serum and urine analyses. To achieve seamless integration and 'hands-free' operation, our assay relies on 1) nanoporous polyacrylamide (PA) immune-filters (12%T) photo-fabricated contiguous with a 2) PA gel electrophoresis (PAGE) separation channel, Figure 1. A 12%T filter was empirically determined to have a pore size cutoff near 150 kDa, making this filter composition relevant to extraction of S100-antibody complexes (161 kDa). Comparison of control (no antibody) and immunosubtraction (antibody present) electropherograms provides assessment of target analyte identity, mobility, and concentration. Unlike most heterogeneous immunoassays, the immunosubtraction assay developed here does not rely on chemical immobilization of subtraction antibody-antigen in the microchannel network. Instead the polymer filter reversibly excludes antibody-antigen complex, making a single channel reusable and, thus, useful to both the control and immunosubtraction assays.

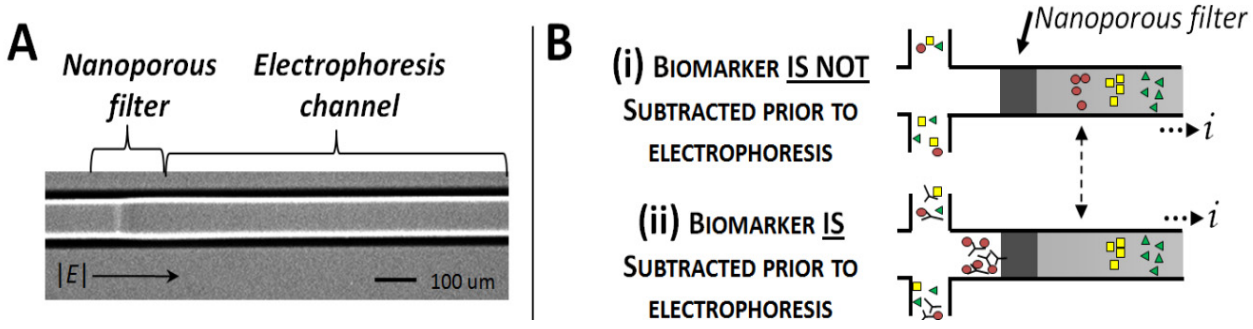

Figure 1: Immunosubtraction is rapid, specific, quantitative and yields a reusable device. (A) Bright field image of subtraction filter and electrophoresis gel in microchannel. (B) If antibody probe binds to protein target, the protein target is "subtracted" from subsequent electrophoresis, as large immune complex is retained at the nanoporous filter prior to electrophoresis. Compare no subtraction case (i) to case with subtraction present (ii).

RESULTS AND DISCUSSION

Successful immunosubtraction of S100 with negative controls is observed via both CCD imaging and single point detection (Figure 2). A sample plug is injected into the PA filter region with 3 µA applied across the filter/separation channel. Migrating protein fluorescence is imaged at 1.5 mm down the separation channel during single point detection measurements. Note the high degree of S100 retention at the nanoporous filter, and the speed of the assay (compared to >6 hrs for some conventional immunosubtraction techniques). Adjusting antibody/target ratio optimizes specific S100 target extraction to achieve ~90% target peak "subtraction" while a non-target protein (ovalbumin) is diminished by ~20% due to filter blockage (Figure 3).

CSF, in particular, would benefit from increased assay automation and throughput owing to the numerous candidates for a TBI detection panel [3]. Thus, building on single biomarker immunosubtraction, dual immunosubtraction for concurrent detection of S100 and C-RP (Figure 3) was completed in < 3 min and yielded facile detection of both putative TBI biomarkers in a single microchannel. The immune-complexes are only temporarily retained at the nanoporous filter, therefore electric field reversal removes the complexes and yields a device easily "refreshed" for subsequent assays.

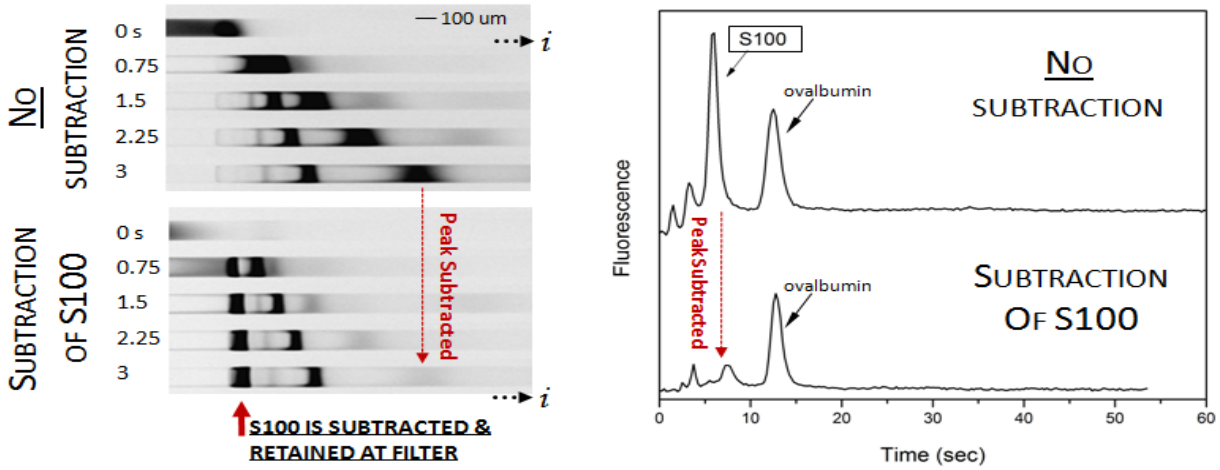

Figure 2: S100, a marker of traumatic brain injury, is immunosubtracted quickly and specifically using the nanoporous filter and antibody system. (Left) CCD images of electrophoresis showing immunosubtraction of S100 in 1X Tris/Glycine buffer. "i" is direction of current flow. (Right) Electropherograms of immunosubtraction in buffer from single-point imaging at 1.5 mm in < 60 s.

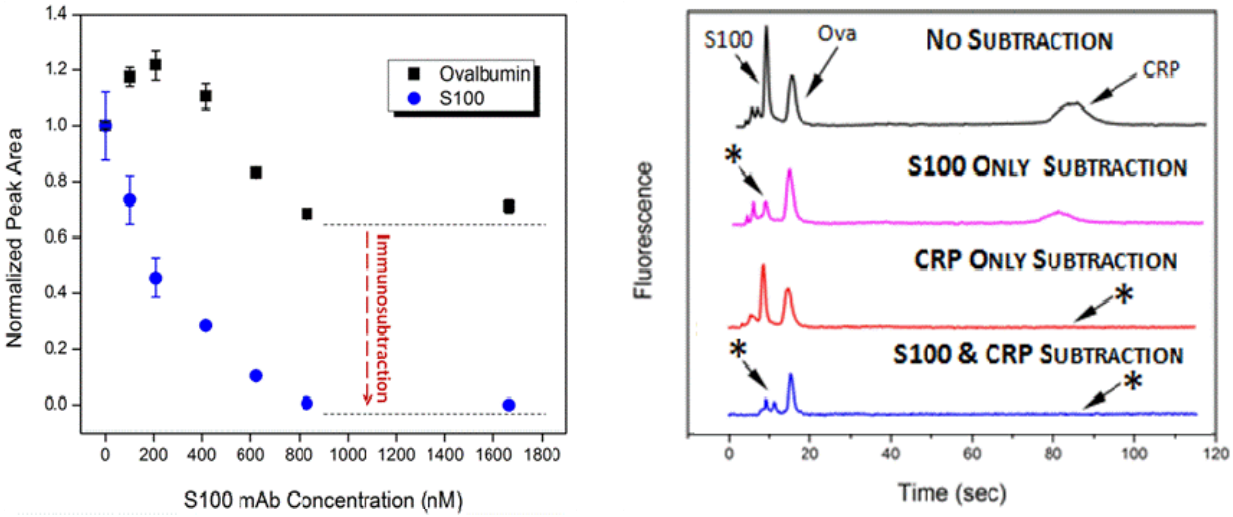

Figure 3: Quantitation of TBI biomarkers S100 and CRP in 1X Tris/Glycine buffer system. (Left) Optimization of size exclusion performance in high-specificity S100 subtraction, as is important for quantitation of S100 level. (Right) Selective subtraction of C-reactive protein (CRP), S100, or both with negative control (ovalbumin) present. "" marks subtracted peaks, which are unique to each immunosubtraction run (i.e., S100 only, CRP only, S100 and CRP).*

Exogenous S100 protein was spiked into unprocessed human CSF at a clinically relevant level (65 nM S100). Both negative control and successful immunosubtraction of the S100 are shown in Figure 4. The large concentration of confounding matrix proteins (0.15 to 0.45 mg/ml) within CSF does not impede the specificity of detecting S100 (0.715 µg/ml) at an elevated concentration indicative of spinal cord injury [4]. The knowledge of S100 mobility allows rapid, specific detection via quantifying subtracted peak area without the need to identify all CSF matrix proteins; however sizing analysis performed with the Agilent Bioanalyzer system indicates the probable identities of Peak 1 and Peak 2 as transferrin (55 kDa) and albumin (66 kDa) respectively. The assay required 2 min and resulted in a signal-to-noise ratio of ~20, which yields a clinically relevant lower limit of detection (3.25 nM).

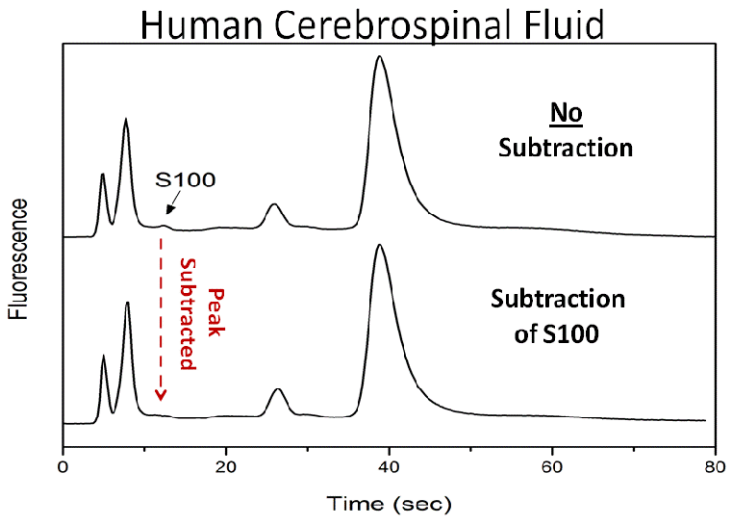

Figure 4: Raw CSF subjected to S100 immunosubtraction results in high specificity identification of S100 at clinical concentrations (65 nM) relevant to traumatic brain injury (1:4 dilution S100 to CSF). Immunosubtraction is performed by incubating the S100/CSF sample with S100 antibody (333 nM). This results in 70% specific subtraction of the S100 peak compared to the control with negligible extraction of non-target proteins (Peak 1 and Peak 2).

CONCLUSION

Our study utilizing a re-useable microfluidic device for rapid immunosubtraction in raw human CSF has demonstrated specific and quantitative detection of multiple analytes in a single run. Optimization of antibody-antigen ratio and applied separation current resulted in the ability to immunosubtract up to 95% of target analyte while extraction of non-target proteins did not reach below 35% for single analyte detection in a model system. A clinically relevant level of S100 was detected from a spiked human CSF sample in < 2 min suggesting the potential capability of the technique as a rapid and accurate means to assess putative protein markers of traumatic brain injury. In related work, we are developing immunosubtraction for detecting monoclonal gammopathies [5]. Future work focuses on extending the multiplexing capabilities, on-chip enrichment, and on-line sample labeling to improve device sensitivity and enable robust TBI diagnostics that confront the enormous challenges in early TBI detection.

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