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Homogeneous immunosubtraction integrated with sample preparation is enabled by a microfluidic format

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Abstract

Immunosubtraction is a powerful and resource-intensive laboratory medicine assay that reports both protein mobility and binding specificity. To expedite and automate this electrophoretic assay, we report on advances to the electrophoretic immunosubtraction assay by introducing a homogeneous, not heterogeneous, format with integrated sample preparation. To accomplish homogeneous immunosubtraction, a step-decrease in separation matrix pore-size at the head of a polyacrylamide gel electrophoresis (PAGE) separation channel enables 'subtraction' of target analyte when capture antibody is present (as the large immune-complex is excluded from PAGE), but no subtraction when capture antibody is absent. Inclusion of sample preparation functionality via small pore size polyacrylamide membranes is also key to automated operation (i.e., sample enrichment, fluorescence sample labeling, and mixing of sample with free capture antibody). Homogenous sample preparation and assay operation allows on-the-fly, integrated subtraction of one to multiple protein targets and reuse of each device. Optimization of the assay is detailed which allowed for ~95% subtraction of target with 20% non-specific extraction of large species at the optimal antibody-antigen ratio, providing conditions needed for selective target identification. We demonstrate the assay on putative markers of injury and inflammation in cerebrospinal fluid (CSF), an emerging area of diagnostics research, by rapidly reporting protein mobility and binding specificity within the sample matrix. We simultaneously detect S100B and C-reactive protein, suspected biomarkers for traumatic brain injury (TBI), in ~2 min. Lastly, we demonstrate S100B detection (65 nM) in raw human CSF with a lower limit of detection of ~3.25 nM, within the clinically relevant concentration range for detecting TBI in CSF. Beyond the novel CSF assay introduced here, a fully automated immunosubtraction assay would impact a spectrum of routine but labor and time-intensive laboratory medicine assays.

Keywords

Immunosubtraction; S100; Microfluidic; Electrophoresis; Homogeneous assay; Cerebrospinal fluid; Trauma; Sample preparation

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SUPPORTING INFORMATION PARAGRAPH

Supporting Information (SI) available: [S-2. Optimizing the selectivity of the homogeneous format. Figure S-1. Integrated sample preparation and immunosubtraction chip layout and protocol. Figure S-2. Detection of immune-complex via excluded fluorescence measurements at the filter interface. Figure S-3. Optimization of immunosubtraction assay to maximize linear range and signal specificity. Figure S-4. Signal enhancement factor of proteins with on-chip enrichment at sample preparation membrane. Table S-1. Peak area for multi-analyte detection.]

Introduction

Immunosubtraction is a widely used laboratory medicine assay often employed to quantify diagnostic proteins by antibody-based bead capture and 'subtraction' of target in subsequent native slab-gel polyacrylamide gel electrophoresis[1–3] (PAGE). Target proteins are identified via comparison of PAGE electropherograms – with and without target extraction[4, 5]. Thus, two electrophoretic assays comprise an immunosubtraction analysis, which is currently the gold-standard for laboratory assessment of monoclonal gammopathies[6]. As is often the case with laboratory medicine assays, bottlenecks stem from both the sample preparation and assay readout steps needed to complete the immunosubtraction analyses.[7] Several specific steps complicate automated and rapid completion of an assay; specifically: sample enrichment to extend the lower limits of detection, sample staining to enable fluorescence readout, and sample mixing with capture antibody reagents to subtract protein targets from subsequent electrophoretic analysis. Recently, a commercial capillary format has been introduced to clinical laboratory medicine, yet the format incorporates a limited selection of monoclonal proteins and does not provide one automated workflow[3, 5, 6, 8, 9]. Nevertheless, immunosubtraction offers benefits over protein electrophoresis, ELISA, and immunostaining techniques as the assay reports target identity through both mobility (charge-to-mass ratio) and immunoaffinity, making the assay highly specific[10].

Recent advances in microfluidic integration of sample preparation with electrophoretic assays for biomarker detection surmount the bottlenecks associated with bench-top slab-gel and capillary techniques[11–14]. In particular, use of microfabricated molecular weight cut-off filters in electrophoresis microchannels allows exclusion and confinement of protein targets[11, 12], as is relevant to subsequent sample manipulation needed for efficient, low sample-loss preparation strategies. Development of nonuniform in-situ fabricated polyacrylamide (PA) gels for ultra-short separation distance homogeneous electrophoretic immunoassays[15] provides a design framework for integration of assay stages in one monolithic, voltage-programmable microdevice useful for laboratory medicine and clinical chemistry.

Immunosubtraction provides binding selectivity and analyte mobility measurements necessary for validating key high diagnostic value biomarkers even in non-systemic proximal fluids. An analytical technology for longitudinal measurements of putative protein biomarkers may facilitate monitoring and validation of promising, yet unverified, markers of dysfunction and injury[16–19]. Increased levels of the protein S100B in CSF have been linked to cellular-level brain injury in multiple sclerosis, meningitis, subarachnoid hemorrhage and cerebral infarction[20]. S100B is in a family of calcium-modulated proteins involved with the regulation of cellular functions and associated with development, function, and disease of the nervous system[21]. Due to low concentrations and a short half-life, S100B protein is difficult to detect and monitor in the systemic circulation[22, 23]. While not routinely collected as a diagnostic fluid, CSF is collected in severe cerebral trauma cases (traumatic brain injury, TBI) when patient cognitive and motor response are impaired (indicated by a Glasgow Coma Score 8 or lower)[24]. In severe trauma cases, CSF collection is performed using external ventricular drainage (ventriculostomy) as a means to reduce intracranial pressure (acute TBI), continuously monitor intracranial pressure, and insert antibiotics directly into the CSF; all of which can be critical to preventing brain damage or death[25]. CSF is also collected to divert blood-contaminated CSF following hemorrhage or neurosurgery[26, 27]. The current standard for assessment of S100B levels in collected CSF relies on labor intensive and slow diffusion based techniques including immunoradiometric assays and sandwich enzyme immunoassays[19, 20, 28, 29]. Fine time-point monitoring would provide rich and currently missing information for biomarker validation, as well as the

potential to monitor therapeutic efficacy (i.e., ventriculostomy) during treatment in cases of severe TBI.

Here we develop an automated microfluidic immunosubtraction format for the first time as a means for rapid protein quantitation in human CSF. On-chip homogeneous immunosubtraction provides advantages over macroscale electrophoretic assays including increased automation, more rapid analysis times, lower sample volume, reduced cost and increased sensitivity[11, 12, 30, 31]. Furthermore, here we present: 1) development of a fully homogeneous format as an alternative to the typically partially heterogeneous format, 2) integration of the flexible format with three key on-chip preparatory steps and 3) identification of CSF trauma biomarkers via quantitative measurements of target mobility and binding specificity. Use of a discontinuous PA gel (3.5%T/12%T) establishes size-based filtration that requires no chemical immobilization or permanent fixation of the target subtraction antibody (Ab) prior to sample electrophoresis. Both the sample preparation and the immunosubtraction assays conducted using the new device are characterized to establish optimal assay conditions. Lastly, the integrated preparatory and analytical tools are used for detecting putative TBI protein markers from raw human cerebrospinal fluid (CSF). The study also suggests the usefulness of the integrated immunosubtraction tool to automate other complex laboratory medicine techniques.

Materials and Methods

Reagents and Proteins

Solutions of 30% (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)propyl methacrylate (98%), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). The water soluble photoinitiator 2,2′-azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086) was purchased from Wako Chemicals (Richmond, VA). Premixed 10 -Tris-glycine native electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine) was purchased from Bio-Rad (Hercules, CA). Recombinant S100 beta protein (S100B), mouse monoclonal antibody to S100B (S100B antibody) and mouse monoclonal antibody to C-reactive protein (CRP antibody) were purchased from Abcam (Cambridge, MA). CRP purified from human serum was purchased from CalBiochem/EMD (Gibbstown, NJ). Ovalbumin (OVA, AlexaFluor 488 conjugated), Carbonic Anhydrase (CA), Chicken Serum Albumin (CSA), Trypsin Inhibitor (TI) and Immunoglobulin G (IgG) were purchased from Invitrogen (Carlsbad, CA). Human cerebrospinal fluid from pooled samples of healthy individuals was purchased from Biological Specialty Corp. (Colmar, PA). Unlabeled proteins were fluorescently labeled in-house using Alexa Fluor 488 protein labeling kits (Life Technologies, Carlsbad, CA). Purification of labeled proteins was performed with P-6 and P-30 Bio-Gel columns (Bio-Rad, Hercules, CA). Post-labeling, proteins were diluted with 1 Tris/Glycine native buffer to attain desired concentrations. On-chip labeling was conducted using reagents from the Quant-iT Protein Assay Kit from Invitrogen Molecular Probes (Eugene, OR). The Quant-iT dye reagent was mixed with 1 Tris/Glycine buffer at a ratio of 1:4. For CSF experiments, S100B and CSF were labeled separately prior to adding the desired concentration of S100B to the CSF sample. Proteins were stored at 4°C in the dark until use. For off-chip preparation, protein-antibody complexes were formed by incubating the target protein with the relevant antibody for at least 1hr at room temperature. Sizing assays of biological samples were performed with the Bioanalyzer 2100 using the standard protein sizing kit (Agilent, Santa Clara, CA).

Chip Fabrication & Surface Preparation

Commercially available chip formats were selected to foster translation of the homogenous immunosubtraction assay to laboratory medicine and clinical labs. Two formats of soda-lime

glass microfluidic chips were purchased from Caliper Life Sciences (Hopkinton, MA). A T-junction geometry was used for the homogeneous immunosubtraction assay characterization. Two intersecting channels terminated in fluid wells denoted here as sample (S), sample waste (SW), buffer (B), and buffer waste (BW). A separation channel was 2.5 mm in length, ~80 μm wide, and 20μm deep. For the integrated sample preparation concatenated with the homogeneous immunosubtraction a more complex channel network was employed (see Figure S-1). This integrated chip consisted of the four wells used for immunosubtraction plus three additional reagent wells used for dye, antibody, and wash buffer all connected to a separation channel that was 2.05 mm in length, ~80 μm wide, and 15μm deep.

Channel walls were prepared for in-situ gel polymerization by washing with 1M NaOH for 5 min to remove debris from the channels followed by a wash with deionized water to remove the NaOH. This was followed by channel wall functionalization to enable covalent linkage to PA with a 2:3:5 ratio solution of 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and deionized water loaded via capillary action. After 30 min the surface preparation solution was vacuum purged and channels were flushed twice with 30% glacial acetic acid followed by two washes with deionized water.

Immunofilter Fabrication & Function Contiguous with Electrophoretic Sieving Gel

A step decrease in PA gel pore-size at the start of an electrophoretic separation channel defines a molecular weight cut-off filter and electrophoretic sieving gel. During electrophoretic analysis of a protein sample, the cut-off filter acts as an immunofilter, as bound capture antibody and protein target are excluded from entering the separation gel. Immunofilters were fabricated from in-situ photo-patterned PA gel precursor solutions made by diluting the total volume of the 30%T (w/v) acrylamide/bisacrylamide solution to the desired ratio and adding 0.2% (w/v) VA-086 photoinitiator. All PA gel precursor solutions were sonicated and degassed 5–10 min prior to loading into channels for photo-polymerization. The molecular weight cut-off filter consisted of a 12%T (total acrylamide %T, with 3.3% w/w cross-linker, %C) PA region. 12%T filters were empirically determined to have a pore-size cutoff near 150 kDa, making this filter composition relevant to subtraction of S100B-antibody complexes. Adjacent to the filter a 3%T loading gel was polymerized creating a step discontinuity in the gel from large to small pore-size between the loading gel and filter region, allowing sample stacking at the interface for improved resolution and unbiased protein loading. Filters were fabricated via photolithography using a film transparency mask with a 500 μm 4 mm opening (Fineline Imaging, Colorado Springs, CO). The open region of the mask was aligned with the desired region of the separation channel containing 12%T precursor solution then exposed to UV power (~5.8 mW/cm^2) for 4 to 5 min. UV illumination was accomplished via a 100W mercury arc lamp and a 4 UV objective on an Olympus IX-50 microscope (Melville, NY) in conjunction with neutral density filters to achieve the desired power. After photo-patterning of the filter, the unpolymerized 12%T precursor solution in the loading channels was exchanged with 3%T PA precursor solution through a vacuum purge. Using a UV lamp in order to expose the entire surface area of the microfluidic channel geometry, the unmasked chip was then subjected to flood illumination for 10 minutes at a distance of 18 cm from the 100W UV lamp (UVP B100-AP, Upland, CA) at ~10 mW/cm^2.

Homogeneous Electrophoretic Immunosubtraction

To initiate electrophoretic immunosubtraction, sample was loaded onto the chip by applying a 1.5 μA current (~300 V/cm) between S and SW, while B and BW were maintained at 0 μA current. For sample injection to the PA filter, 3 μA (~300 V/cm) was applied between B and BW, while S and SW were maintained at 0 μA (floating potential). For baseline cases in

which no antibody was present, sample proteins were mixed just prior to performing separations. For the immunosubtraction cases with off-chip sample preparation, target protein was incubated for one hour with the relevant antibody prior to loading. The immunosubtraction cases conducted with on-chip sample preparation are detailed in the Results & Discussion section. A minimum of three replicate runs for each sample were performed. After each assay, protein complexes excluded at the filter were removed by reversing the electrical potential to apply 3 μA (~300 V/cm) from BW to SW, while B and S were maintained at 0 μA (floating potential). Channels were electrophoretically flushed with 1 Tris/Glycine buffer prior to each new assay. To generate electropherograms, the separation channel was imaged at 1.5 mm downstream of the separation junction. The channel was also imaged at the filter interface (250 μm downstream of the separation junction) to yield measurements of total excluded complex fluorescence. Total assay time including sample loading, separation, and removal of sample from the filter was ~5 min per run. IgG assays to investigate non-specific filter blockage and antibody cross-reactivity were run using the same conditions described above for immunosubtraction assays. Increasing concentrations of IgG (6 nM to 5 μM) were incubated for 1 hr with S100B (234 nM) prior to running on-chip immunosubtraction.

Sample Preparation Membrane Fabrication & Functions

A sample preparation membrane was fabricated in-situ with a photo-polymerization process similar to the immunosubtraction filter. A 40%T 6%C acrylamide/bisacrylamide solution with 0.2% VA-086 photoinitiator was sonicated and degassed for 5 min then introduced into the microfluidic channels. To photo-polymerize the sample preparation membrane, a sheet of UV light from the high intensity laser was used to: 1) localize the feature in the microchannel network and 2) yield sharp, well-defined boundaries. The sample preparation membrane was photo-polymerized with a 2 kW 355 nm Nd:YAG laser (Teem Photonics, Lafayette, CO) directed through cylindrical focusing optics and a 100 μm slit in a chrome on glass mask (Photo Sciences, Torrance CA) for 75 s. This resulted in a ~100 μm wide PA sample preparation membrane with a pore size sufficient to prevent passage of S100 (11 kDa) and all larger proteins while allowing free dye to pass through. The 3%T loading gel and 12%T immunosubtraction filter were fabricated adjacent to the 40%T sample preparation membrane in the manner described previously. The sample preparation membrane was used for enrichment of proteins and mixing of samples with required reagents for on-chip labeling and binding at discrete intervals. Samples and reagents were both loaded to the membrane and eluted by applying 0.1 μA (1100 V/cm) across the appropriate current path followed by subsequent immunosubtraction as described previously (see Figure S-1).

Apparatus and Imaging

Glass chips were seated in a custom Delrin manifold to provide sample reservoirs for each well. 5 μL of sample was loaded into the sample well while all other wells were filled with 60 μL 1 Tris/Glycine native buffer. Electrophoretic loading of sample was performed with a custom-built, programmable high-voltage power supply. Platinum wire was used as electrodes. Imaging was performed with inverted epi-fluorescence microscopes (IX-70, Olympus, Melville, NY & Diaphot 200, Nikon Instruments, Melville, NY). Images were collected with a 10 objective (NA 0.3), using a filter cube optimized for GFP detection and a 0.63 demagnifier (Diagnostic Instruments Inc., Sterling Heights, MI). A 139 040 Peltier-cooled interline CCD camera (CoolSNAP HQ2, Roper Scientific, Trenton NJ) was used to record images of the protein migration. An image exposure time of 250 ms was used with 2 pixel binning applied in the y-direction (transverse to separation). Image analysis was performed using Image J software (National Institutes of Health, http://rsbweb.nih.gov/ij/). All images were background subtracted to account for variability in background signal.

Electropherograms were generated by measuring fluorescence intensity in a region of interest (ROI) occupying the width of the channel at a single axial position (ROI ~ 160 μm 80 μm) ~1.5 mm downstream from the immunosubtraction filter interface. Variations in signal intensity due to CCD noise or external light fluctuation were normalized with the recorded signal to either a free-dye internal standard or total fluorescence of the injected sample in the separation channel.

Results and Discussion

Homogenous Electrophoretic Immunosubtraction

The on-chip immunosubtraction assays consisted of two electrophoretic separations: 1) a baseline sample separation in which no capture antibody was present and 2) a subsequent separation after capture antibody was incubated with the sample (Figure 1). Importantly, the immunosubtraction assay described here did not employ chemical immobilization of subtraction antibody in the microchannel network. Contiguous to the separation channel was a small pore-size membrane that allowed integration of several sample preparation functions needed to implement immunosubtraction in an automated manner. After sample preparation, both sample aliquots were serially injected into the separation channel which contained a PA sieving gel. A sharp step discontinuity from large-to-small pore-size gel was located at the start of the PA sieving gel, somewhat similar to a stacking gel in a slab gel system. This immunosubtraction filter reversibly excluded antigen-antibody complex. Thus, when both protein target and capture antibody were present, the resulting large immune-complex was excluded from entering the PAGE separation region. Consequently, the target analyte peak was not present in the PAGE assay. When no capture antibody was present, the sample contained all protein peaks. Comparison of a baseline assay (Ab not present) and an immunosubtraction assay (Ab present) resulted in an apparent “subtraction” of the target peak. All assays were conducted under native (non-reducing, non-denaturing) conditions, to preserve the affinity of target protein to antibody.

As a proof-of-principle for the homogeneous immunosubtraction assay, analysis of S100B protein (208 nM) with matrix surrogate ovalbumin (49 nM) was conducted. A simplified matrix was initially studied to allow controlled increase of the concentration of a major confounding protein. Figure 2 presents comparative baseline and immunosubtraction data. The separations showed that when unlabeled S100B antibody (693 nM) was added to the sample, the peak area of free S100B in subsequent PAGE was notably reduced. At the same time, full field imaging showed size-based exclusion of large S100B-antibody complexes at the 12%T immunosubtraction filter interface. As the assay was developed to measure the low molecular weight S100B protein (11 kDa), the 12%T immunosubtraction filter pore-size was optimized to prevent passage of proteins larger than ~150 kDa (e.g., the S100B-antibody complex at 161 kDa) while permitting passage of proteins smaller than the pore-size cutoff including the unbound S100B and ovalbumin (45 kDa). Although the immunosubtraction filter allowed unambiguous immunosubtraction of the target protein, full subtraction of the target peak was not necessary for target identification. Figure 2 further reveals minimal effects of immunosubtraction on the ovalbumin peak area or mobility, as ovalbumin was able to traverse the immunosubtraction filter interface. As the 12%T immunosubtraction filter was present in both the baseline and immunosubtraction assays, comparison of the baseline to the assay was effective in identifying specific proteins smaller than ~150 kDa that were subtracted via antibody interaction. The immunosubtraction filter pore-size can be adjusted to accommodate a wide range of size cut-off points.

Under the conditions described for the model system, the lower limit of detection (LLOD) for S100B was 1.1 nM with the epi-fluorescence microscope and CCD imaging system used. LLOD decreased to 193 pM for detecting S100B binding exclusively via imaging of

excluded protein at the filter interface (see Figure S-2). The LLOD was within the clinically relevant concentration range of S100B in TBI and is especially relevant to monitoring of increasing S100B levels post-trauma. S100B in CSF can reach levels up to 65 nM (26 nM S100B mean concentration) 48 hours after spinal cord injury[19]. The immunosubtraction assay reported the apparent electrophoretic mobilities of the S100B protein and ovalbumin as 1.1×10^{-8} m^2/Vs and 3.5×10^{-9} m^2/Vs, respectively. Under the PAGE conditions employed, baseline separation of the two peaks in the model system was achieved at 1.5 s and in a separation length of 0.4 mm.

After assay completion, the application of a reversed polarity electrical potential (300 V/cm) across the immunosubtraction filter was used to remove residual or subtracted proteins from both the filter and separation channel, as a means to regenerate the channel for subsequent assays. As mentioned, no irreversible or chemical immobilization of the antibody was employed, making reuse of the filter for both the baseline and immunosubtraction assays feasible. Figure 2C reports the fluorescence signal at the immunosubtraction filter upon first immunosubtraction separation (run 1) and after electrophoresing away excluded protein. The fluorescence images in Figure 2C show no notable residual protein on or in the filter after 55 runs, nor reduced performance of the cleaning step. As shown, the replicate runs did not result in significant permanent protein immobilization allowing the device to be used for more than 100 runs before eventual degradation of the sieving matrix. Some permanent non-specific fouling of the filter was eventually observed with other assays; thus, the reusability of the filter for a given assay was ultimately dependent upon several factors including sample protein concentration, extraction antibody concentration, discontinuous gel %T, and applied electric field.

Optimization of the immunosubtraction assay was performed on a model sample containing matrix protein (ovalbumin, 2.75 μM) at a molar concentration 26x greater than the target analyte (S100B, 104 nM) in order to mimic high concentration background proteins while maintaining control over concentrations of those proteins (see supporting information, S-2). Optimization of extraction conditions resulted in selective subtraction of 95% of the S100B peak with only 20% non-specific subtraction of ovalbumin (due to filter blockage) at an antibody-S100B molar concentration ratio of 4-to-1. Optimization of the linear range demonstrated that at a fixed antibody-S100B molar concentration (2.5-to-1) the linear range of detection was 30 pM to 1μM, which encompasses the expected levels of S100B in CSF indicative of traumatic brain injury (see Figure S-3).

Immunosubtraction of S100B from Raw Human CSF

To characterize performance of the on-chip immunosubtraction protocol for a minimally processed human diagnostic fluid, exogenous and fluorescently labeled S100B was spiked into raw diluted (4:1) whole CSF. Electrophoretic separation of an S100B spiked CSF sample resulted in three major peaks, with two additional fast moving free dye peaks (Figure 3). Apparent electrophoretic mobilities of the major species were: S100B at 8.5×10^{-9} m^2/Vs, Peak 1 at 4.1×10^{-9} m^2/Vs, and Peak 2 at 2.8×10^{-9} m^2/Vs. Protein sizing indicated that Peak 2 (the slowest peak) had a molecular weight of ~62 kDa, suggesting the peak was human albumin which constitutes 40–60% of CSF protein[32, 33]. The mobility and relative abundance of Peak 1 (compared to albumin) suggested that the peak was likely transthyretin. Transthyretin has a molecular weight of 55 kDa and can comprise up to 25% of total CSF protein[34].

To perform the homogeneous immunosubtraction assay, CSF samples spiked with S100B at 65 nM were incubated with S100B antibody at 333 nM (Figure 3A). Immunosubtraction reduced the S100B peak area to 31% ± 14 of the S100B baseline peak area value. Changes in peak area for the non-target major species were measured as: Peak 1 (transthyretin) at

106% ± 6 and Peak 2 (albumin) at 97% ± 8 of their respective baseline peak area values. These results indicated that non-specific subtraction of non-target protein bands (Peaks 1 and 2) in CSF was negligible. The large concentration of confounding matrix proteins (0.15 to 0.45 mg/ml) in CSF did not impede detection of S100B (0.715 μg/ml) at an elevated concentration indicative of spinal cord or traumatic brain injury[19, 35]. The knowledge of S100B mobility allowed rapid, specific detection via quantifying peak area without the need to identify all CSF matrix proteins. Figure 3A shows the difficulty in visually interpreting subtraction and identity of low concentration target analyte in the presence of large concentrations of confounding proteins, a challenge that can be mitigated through inclusion of on-chip high abundance protein depletion or preparatory protein fractionation steps, as is currently underway.

Multiplexed Homogeneous Immunosubtraction

On-chip immunosubtraction was further developed to simultaneously assay two proteins in one sample, as is relevant to disease biomarker panel analysis. Samples containing S100B (208 nM), ovalbumin (48.6 nM), and CRP (98.6 nM) were analyzed for a capability to subtract both S100B and CRP in one assay (Figure 3B). Samples were incubated off-chip with antibodies to their respective targets (1.11 μM S100B, 725 nM CRP). On-chip immunosubtraction resulted in the baseline assay resolving the three protein standard into three resolved peaks in ~2 min of PAGE. The addition of either S100B or CRP antibody resulted in subtraction of the respective target protein at the filter altering the electropherogram peak profile. The addition of both S100B and CRP antibodies resulted in the simultaneous selective subtraction of both targets (see Table S-1). As demonstrated, 'on-the-fly' target selection was implemented through simple selection and mixing of antibody probes with samples; as no *a priori* surface functionalization is needed prior to assay commencement.

Integration of Preparatory Functions

The integration of multiple sample preparation steps needed to provide a complete and rapid assay that minimized laborious and manual preparatory functions was facilitated by the use of a microfluidic format with in-situ fabricated PA gels. Several discrete PA regions of varying pore sizes formed distinct reaction chambers within the microfluidic channels that provided capability to perform various functions prior to the immunosubtraction assay including: 1) in-line electrophoretic enrichment of low concentration analytes, 2) on-chip fluorescence labeling of samples, and 3) rapid on-chip binding and incubation of antibody and target proteins.

First, enrichment was achieved by electrophoretically loading sample towards a 100 μm wide PA sample preparation membrane (40%T/6%C, Figure 1) in the 7 well chip (see Figure S-1). The small pore size of the 40%T sample preparation membrane allowed the passage of small free dye molecules (640 Da) but prevented passage of all sample proteins, of which S100B (11 kDa) was the smallest. A sample containing model proteins (TI, OVA, and CRP) was enriched at the sample preparation membrane prior to electrophoresis resulting in a total protein enrichment factor of 12 over the baseline in just 2 minutes (Figure 4A). No fluorescence signal was detectable without enrichment prior to electrophoresis, thus 10 s enrichment was used as a baseline value to assess the increase in sensitivity due to enrichment. The protein enrichment, or signal enhancement, factor increased linearly with loading time for the model system explored ($y = 0.099x + 0.346$, $R^2 = 0.992$), with the rate of enrichment of each individual protein dependent upon the electrophoretic mobility as described in previous studies[11, 12] (see Figure S-4). As the system sensitivity, or lower limit of detection, is proportional to the enrichment factor, the LLOD would also be expected to increase directly with enrichment time until reaching the point where concentration

polarization limits effectiveness of continued electrophoretic enrichment[12]. The ability to confine sample at a location—without immobilization—in a homogeneous assay format allowed further manipulation to concentrate the sample downstream while obviating the need for slow and labor intensive bench-top enrichment techniques including vacuum centrifuge and evaporative concentrators.

Next, on-chip fluorescence labeling of samples was achieved via electrophoretic transport and mixing of a fluorogenic dye (Quant-iT) with target proteins at the sample preparation membrane. Quant-iT, a non-covalent binding dye, has minimal fluorescence in free solution however undergoes significant fluorescence enhancement when bound to proteins. Therefore the Quant-iT dye is useful for immunosubtraction assays since all sample proteins are labeled before comparison of a baseline and antibody subtraction PAGE run. Quant-iT dye (1:4, reagent to 1 Tris/Glycine) was initially injected into the loading channel adjacent the sample preparation membrane (see Figure S-1). An unlabeled sample of model proteins (S100B 1.21 μM, CA 1.36 μM, CSA 888 nM) was then injected into the same channel co-locating with the free dye and initiating the labeling interaction. The dye and protein were then electrophoretically transported to the sample preparation membrane confining them into a small reaction region facilitating more efficient binding. Figure 4B shows a linear increase in protein signal with labeling time following injection of the fluorescently labeled proteins into the separation channel for subsequent PAGE ($y = 0.284x - 0.168$, $R^2 = 0.989$). Labeling with Quant-iT for 10 s resulted in approximately 4 increase in detectable signal for each sample protein (S100B 4.1, CA 4.3, CSA 4.1). Including necessary reagent loading and transport steps, on-chip fluorescence labeling was achieved in 70 s compared to the off-chip labeled assays which required anywhere from 1 hr to overnight to incubate and prepare samples for covalent labeling with AlexaFluor 488 dye.

Finally, with the goal of integrating all required preparatory functions into the immunosubtraction assay, on-chip antibody binding and incubation were incorporated at the sample preparation membrane. CRP antibody (4.4 μM) was electrophoretically introduced to the sample preparation membrane where previously enriched model proteins were present (TI 200 nM, OVA 97 nM, CRP 393 nM; proteins labeled off chip). Continuous introduction of antibody increased antibody-antigen binding and removal of the target analyte during subsequent immunosubtraction PAGE analysis (Figure 4C). Results showed the rapid immunosubtraction of 69% of the CRP peak with only 30 s of antibody binding time. Further increase in antibody binding time resulted in more modest improvement, achieving maximum target extraction of 78 % CRP with an antibody binding time of 120 s. Since co-location of antigen and antibody was electrophoretically driven in this homogeneous system, binding occurred faster than in diffusion based systems with no separate incubation step required. Antibody immobilized on surface substrates or Sepharose beads typically requires at least 30 minutes of incubation with the antigen while standard off-chip free solution incubation requires at least an hour to achieve maximum binding—thus the on-chip sample preparation format achieved antibody binding and incubation an order of magnitude faster. Programmable on-chip binding also afforded the flexibility to rapidly establish ideal antibody-antigen ratios to maximize target extraction while providing multiplexing capabilities by introducing distinct antibodies specific for individual targets simultaneously or within consecutive assay runs.

Conclusions

The on-chip homogeneous immunosubtraction introduced here yielded detection of target analytes via rapid PAGE to provide data on mobility and specificity of target molecules. Measured LLOD for determining specificity and mobility of S100B in a model system was 1.1 nM, with detection of binding specificity achievable down to 193 pM. Estimated LLOD

for S100B to assess TBI in raw human CSF was 3.25 nM. Optimization of antibody-antigen ratio and applied separation current resulted in the ability to immunosubtract up to 95% of target analyte with only 20% non-target protein extraction for single analyte detection in a model system. While specific and significant reduction of the target was required to identify the analyte, full subtraction was not necessary. Consequently, on-chip immunosubtraction can be optimized to maximize specificity of subtraction.

The on-chip immunosubtraction device was used for detection of S100B (65 nM) spiked into a pooled sample of human cerebrospinal fluid in under 2 minutes using 5 μl of sample. This suggests potential capability as a rapid means to assess putative protein markers of traumatic brain injury. Detection in the biological sample also proved to be very selective as subtraction of non-target proteins was negligible. Introduction of sample preparation functions on-chip including protein enrichment, labeling, and antibody-binding were shown to significantly reduce labor intensive effort and time required for the total assay including preparatory functions from 4–12 hours to 5–10 min[3, 4, 7, 36]; while also improving system sensitivity 12. Performing immunosubtraction on-chip in an automated and programmable manner on a uniform platform opens up the application space to detecting numerous biomarkers for various proximal fluid diagnostics, as well as for serum and urine analyses. Currently, studies are underway to include on-chip fractionation and removal of high abundance matrix proteins to further improve assay detection sensitivity and reduce sample pre-processing steps.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Caton, JE.; Holladay, DW.; Anderson, NG. Abstracts of Papers of the American Chemical Society. 1973. p. 31
2. Keren DF. Archives of Pathology & Laboratory Medicine. 1999; 123:126–132. [PubMed: 10050785]
3. Keren, DF. Protein Electrophoresis in Clinical Diagnostics. Arnold Publishers; 2003.
4. Bossuyt X, Bogaerts A, Schiettekatte G, Blanckaert N. Clinical Chemistry. 1998; 44:760–764. [PubMed: 9554486]
5. Paquette DM, Banks PR. Electrophoresis. 2001; 22:2391–2397. [PubMed: 11519941]
6. Hutchison CA, Basnayake K, Cockwell P. Nature Reviews Nephrology. 2009; 5:621–627.
7. Clark R, Katzmann JA, Kyle RA, Fleisher M, Landers JP. Electrophoresis. 1998; 19:2479–2484. [PubMed: 9820971]
8. Marshall T, Williams KM. Electrophoresis. 1999; 20:1307–1324. [PubMed: 10424453]
9. Bienvenu J, Graziani MS, Arpin F, Bernon H, Blessum C, Marchetti C, Righetti G, Somenzini M, Verga G, Aguzzi F. Clinical Chemistry. 1998; 44:599–605. [PubMed: 9510868]
10. Aguzzi F, Poggi N. Boll Ist Sieroter Milan. 1977; 56:212–216. [PubMed: 901635]
11. Herr AE, Hatch AV, Throckmorton DJ, Tran HM, Brennan JS, Giannobile WV, Singh AK. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:5268–5273. [PubMed: 17374724]

12. Hatch AV, Herr AE, Throckmorton DJ, Brennan JS, Singh AK. Analytical Chemistry. 2006; 78:4976–4984. [PubMed: 16841920]
13. Sun XH, Yang WC, Pan T, Woolley AT. Analytical Chemistry. 2008; 80:5126–5130. [PubMed: 18479142]
14. Beyor N, Yi LN, Seo TS, Mathies RA. Analytical Chemistry. 2009; 81:3523–3528. [PubMed: 19341275]
15. Hou CL, Herr AE. Analytical Chemistry. 2010; 82:3343–3351. [PubMed: 20334346]
16. Thorngren-Jerneck K, Alling C, Herbst A, Amer-Wahlin I, Marsal K. Pediatric Research. 2004; 55:406–412. [PubMed: 14630979]
17. Tsakadze NL, Shakarishvili RR, Akhvlediani KS, Chikobava E, Sukoyan G, Karsanov NV. Journal of Neurochemistry. 1998; 71:S72–S72.
18. Infante JR, Torres-Avisbal M, Martinez A, Vallejo JA, Aguilera C, Contreras P, Benitez A, Latre J. Tumor Biology. 2000; 21:38–45. [PubMed: 10601840]
19. Kunihara T, Shiiya N, Yasuda K. The Journal of Thoracic and Cardiovascular Surgery. 2001; 122:1019–1020. [PubMed: 11689809]
20. Nygaard O, Langbakk B, Romner B. Clinical Chemistry. 1997; 43:541–543. [PubMed: 9068602]
21. Zimmer DB, Cornwall EH, Landar A, Song W. Brain Res Bull. 1995; 37:417–429. [PubMed: 7620916]
22. Mussack T, Klauss V, Ruppert V, Gippner-Steppert C, Biberthaler P, Schiemann U, Hoffmann U, Jochum M. Clinical Biochemistry. 2006; 39:349–356. [PubMed: 16460721]
23. Ghanem G, Loir B, Morandini R, Sales F, Lienard D, Eggermont A, Lejeune F. Int J Cancer. 2001; 94:586–590. [PubMed: 11745448]
24. Sharples PM, Stuart AG, Matthews DS, Aynsley-Green A, Eyre JA. J Neurol Neurosurg Psychiatry. 1995; 58:145–152. [PubMed: 7876842]
25. Hickman, JL. Critical Care Neurology and Neurosurgery. Suarez, JI., editor. Humana Press Inc; Totowa, NJ: 2004. p. 571-580.
26. Woodward S, Addison C, Shah S, Brennan F, MacLeod A, Clements M. Br J Nurs. 2002; 11:47–53. [PubMed: 11826320]
27. Neilsen, PKN.; Limbacher, E.; Wallace, DC. Nursing Care of the Pediatric Neurosurgery Patient. CCaWD, editor. Springer; Berlin: 2007.
28. Otto M, Stein H, Szudra A, Zerr I, Bodemer M, Gefeller O, Poser S, Kretzschmar HA, Mader M, Weber T. Journal of Neurology. 1997; 244:566–570. [PubMed: 9352454]
29. Hayakata T, Shiozaki T, Tasaki O, Ikegawa H, Inoue Y, Toshiyuki F, Hosotubo H, Kieko F, Yamashita T, Tanaka H, Shimazu T, Sugimoto H. Shock. 2004; 22:102–107. [PubMed: 15257081]
30. Yager P, Edwards T, Fu E, Helton K, Nelson K, Tam M, Weigl B. Nature. 2006; 442:412–418. [PubMed: 16871209]
31. Herr AE, Throckmorton DJ, Davenport AA, Singh AK. Analytical Chemistry. 2005; 77:585–590. [PubMed: 15649057]
32. Irani, D. Cerebrospinal Fluid in Clinical Practice. Saunders Elsevier; Philadelphia: 2009.
33. Kabat EA, Glusman M, Knaub V. American Journal of Medicine. 1948; 4:653–662. [PubMed: 18856761]
34. Serot JM, Christmann D, Dubost T, Couturier M. Journal of Neurology, Neurosurgery & Psychiatry. 1997; 63:506–508.
35. Czernicki, Z.; Baethmann, A.; Ito, U.; Katayama, Y.; Kuroiwa, T.; Mendelow, D.; Kleindienst, A.; Meissner, S.; Eyupoglu, IY.; Parsch, H.; Schmidt, C.; Buchfelder, M. Brain Edema. Vol. XIV. Springer; Vienna: 2009. p. 247-250.
36. Palfrey, S. Clinical Applications of Capillary Electrophoresis. Palfrey, S., editor. Humana Press; Totowa, NJ: 1999. p. 39-46.

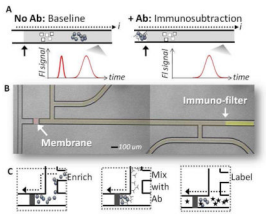

Figure 1. Sample preparation is integrated with downstream PAGE for protein immunosubtraction electrophoresis in a homogeneous format

(A) The PAGE pore-size discontinuity (stacking gel) acts as an immunosubtraction filter when complexing antibody is present, (+ Ab) thus excluding specific sample peaks from the resultant PAGE electropherogram. Comparison to a baseline electropherogram (No Ab) reveals analyte mobility and interaction. (B) Photo-patterning of contiguous PA gel features having different pore-sizes allows definition of preparative and analytical functions in one monolithic device. (C) The sample preparation membrane is used in conjunction with electrophoretic analyte transport to enable sample enrichment, mixing of sample with antibodies, and fluorescence labeling of the sample (see Figure S-1 for detail).

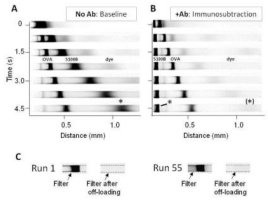

Figure 2. Immunosubtraction is accomplished in a purely homogeneous on-chip assay

(**A**) A baseline PAGE separation, i.e., with no complexing antibody present, of a three component model sample shows rapid assay completion in a short separation distance (<1 mm). Sample components include a positive control (S100B, marked with *), a negative control (ovalbumin), and a fast free dye peak (Alexa Fluor 488). (**B**) Subsequent to baseline analysis, the same assay is performed now with unlabeled antibody against S100B present, allowing determination of the S100B peak identity. With S100B antibody present, significant S100B mass is retained at the immunosubtraction filter (*). (**C**) After immune-complex subtraction via retention at the immunosubtraction filter, a reverse polarity electric potential is used to drive immune-complex off the immunosubtraction filter and prepare the PAGE gel and immunosubtraction filter for re-use.

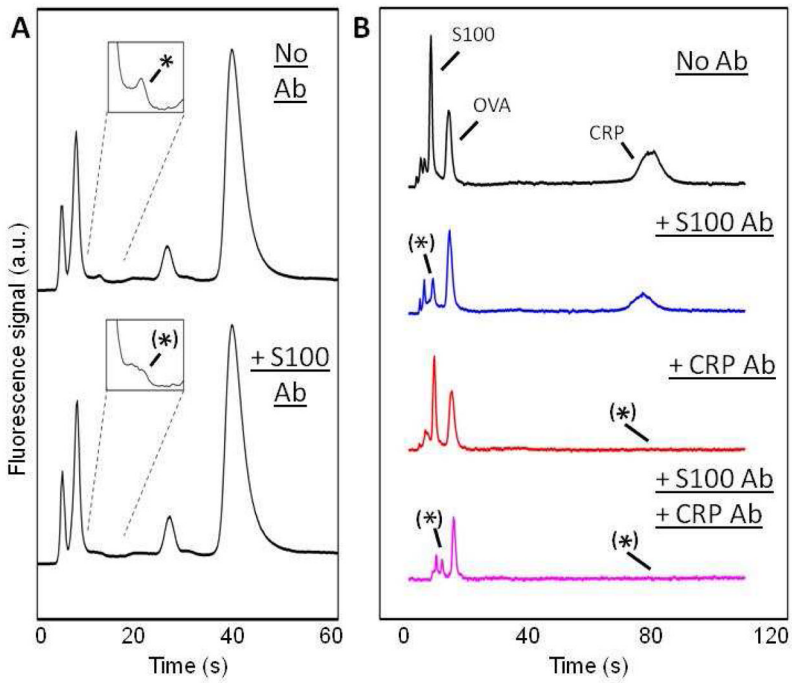

Figure 3. Homogeneous assay enables immunosubtraction of putative protein biomarkers from complex biological fluids and as multi-analyte panels

(A) 65 nM S100B was spiked into pooled human CSF and assessed via on-chip immunosubtraction. Five peaks are resolved, including two fast moving free dye peaks, the S100B peak (*) and two slower mobility peaks determined to be transthyretin and albumin. The addition of antibody against S100B yields specific subtraction of the high mobility S100B peak. (B) A homogeneous format supports multi-analyte subtraction during one PAGE separation. Subtraction and identification of one and two target proteins is illustrated by comparison of the baseline electropherogram to PAGE of samples with antibody present for: S100B, CRP, and both S100B and CRP. The (*) symbol indicates the peak being subtracted from the assay. E = 300 V/cm, detection point at 1.5 mm. All proteins labeled with Alexa Fluor 488.

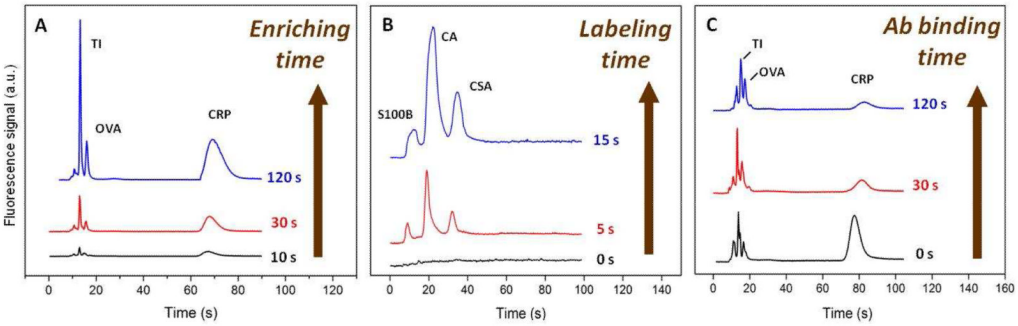

Figure 4. Sample preparation including sample enrichment, on-line fluorescence labeling, and antibody mixing are integrated using a combination of electrophoretic transport and a small pore-size PA gel sample preparation membrane

(A) Sample enrichment at the sample preparation membrane significantly increases enrichment factor of 3 pre-labeled model proteins (TI 200 nM, OVA 97 nM, CRP 393 nM). Protein enrichment increases linearly with increasing electrophoretic loading to the PA membrane resulting in enrichment factors of 18x, 11x, and 7x respectively for TI, CRP, and OVA in under 2 min. (B) Non-covalent fluorescence labeling and enrichment of sample is achieved by co-loading a non-covalent fluorogenic dye (Quant-iT) with model proteins (S100B 1.21 μM, CA 1.36 μM, CSA 888 nM). The signal of each protein increases 4x with 10 s dye/enrichment time due to labeling. (C) On-chip mixing and incubation of pre-labeled sample with antibodies (TI 200 nM, OVA 97 nM, CRP 393 nM). Mixing with antibody against CRP (4.4 μM) is performed just prior to downstream baseline (no Ab, 0 s) and immunosubtraction (+Ab, 30 s and 120 s) PAGE resulting in increased specific CRP target extraction with Ab load/enrichment time (78% extraction at 120 s). E = 1100 V/cm across the sample preparation membrane for all sample preparation steps. E = 200 V/cm separation with imaging at 1.5 mm. In each panel, electropherograms are slightly offset (y-axis) to aid interpretation.