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Review

Clinically relevant advances in on-chip affinity-based electrophoresis and electrochromatography

Clinical and point-of-care disease diagnostics promise to play an important role in personalized medicine, new approaches to global health, and health monitoring. Emerging instrument platforms based on lab-on-a-chip technology can confer perfomance advantages successfully exploited in electrophoresis and electrochromatography to affinity-based electrokinetic separations. This review surveys lab-on-a-chip diagnostic developments in affinity-based electokinetic separations for quantitation of proteins, integration of preparatory functions needed for subsequent analysis of diverse biological samples, and initial forays into multiplexed analyses. The technologies detailed here underpin new clinical and point-of-care diagnostic strategies. The techniques and devices promise to advance translation of until now laborabory-based sample preparation and analytical assays to near-patient settings.

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Biomarker / Clinical diagnostic / Immunoassay / Lab-on-a-chip / Microfluidic
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1 Introduction

A successful transition from curative medicine to predictive, personalized, and even preemptive medicine depends on the availability of multiplexed (multianalyte) point-of-care diagnostic tools. To fully realize a "bench-to-bedside" paradigm, translation of analytical-grade diagnostic tools from centralized laboratory facilities to near-patient settings is needed. Readily accessible, rapid diagnostic assays would be ideally employed routinely and for diverse clinical applications. Examples of target applications include: (i) prediction of disease onset, (ii) stratification of disease (*e.g.*, especially for episodic diseases with "flares" of activity), (iii) indications of disease progression, (iv) guidance of treatment decisions (*i.e.*, identify drug resistance or potential adverse reactions), and (v) monitoring of treatment efficacy. In this review, we detail recent notable advances in clinical diagnostic instrumentation fueled by progress in "lab-on-a-chip" technology [1–3]. Here, we focus on clinically relevant developments in affinity-based electrophoretic and electrochromatographic assays, which we broadly term affinity-based "electrokinetic" assays. Central to protein analysis in complex diagnostic fluids, affinity-based electrokinetic assays are just beginning to benefit from microfluidic technologies. Although still at an early stage, the body of work summarized in this review gives an indication of the future clinical impact potentially conferred through lab-on-a-chip technology.

As with CE and CEC, capillary-based clinical analyses using affinity methods are presently more mature than their microchip-based counterparts. Owing to our present focus on microfluidic technology, we organize the material, generally, in order of increasing instrument sophistication and assay capabilities. For a comprehensive review of important advances in capillary-based technology, we direct the reader to other recent reviews [4–10].

1.1 Affinity-based electrokinetic assays

As noted by Amundsen and Siren [5], nomenclature in the affinity-based electrokinetic assay topical area is varied. In this review, we provide a detailed description of assay opera-

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Abbreviations: Ab, antibody; **Ab*,** labeled antibody; **Ag,** antigen; **Ag*,** labeled antigen; **AFP,** α-fetoprotein; **ALP,** alkaline phosphatase; **BSA*,** labeled BSA; **CL,** chemiluminescence; **CSF,** cerebral spinal fluid; **Fab,** antigen binding antibody fragment; **HRP,** horseradish peroxidase; **IAP,** immunosuppressive acidic protein; **Ins,** insulin; **Ins*,** labeled insulin; **KCE,** kinetic CE; **L3,** LCA-reactive AFP; **LCA,** *Lens culinaris agglutin*; **MMP-8,** matrix metalloproteinase-8; **PSA,** prostate-specific Ag; **T4,** 3,5,3′,5′-tetraiodol-L-throxine; **Th,** theophylline; **Th*,** labeled theophylline

tion and avoid, as much as possible, nonstandarized nomenclature. Nevertheless, the reader may find useful three previously defined categories of affinity-based electrokinetic assays [5, 11]:

(i) Affinity preparative: Affinity reagent is immobilized onto a support material integrated in the separation path, with affinity-based binding occuring before or during the affinity-based electrokinetic separation.

(ii) Pre-equilibrated affinity-based electrokinetic assays: Affinity reagent(s) and sample are mixed in a vessel, allowing complexation to proceed to equilibrium before introducing the mixture into the affinity-based electrokinetic separation.

(iii) On-chip equilibrated affinity-based eletrokinetic assays: Affinity reagent(s) and sample are dynamically mixed in a separation channel, with affinity reagent in the running buffer.

Figure 1 depicts examples of common sample preparation strategies and assay formats. Regardless of nomenclature or implementation, affinity-based assays require both high analytical specificity (low cross-reactivity) and high analytical sensitivity (lower LOD) [12]. Affinity reagents are selected *a priori* to probe for known, putative protein biomarkers in complex biological matrices. Use of well-characterized affinity reagents imparts required analytical specificity. Commonly employed affinity interactions include lectin, immunological (antibody–antigen or Ab–Ag), immobilized metal, sugar, protein A, protein G, aptamer, and enzymatic among others. Detailed information on various affinity reagents can be found in the recent review by Mondal and Gupta [13].

Immunoaffinity assays form the basis for selective assays and are, consequently, a workhorse clinical format. A review of relevant Ab biochemistry and commonly employed Ab-based detection strategies is given by Amundsen and Siren [5]. Two immunoaffinity assay strategies are commonly used, namely direct and competitive immunoassays [14]. As an example, we discuss immunoaffinity electrokinetic assays in the context of fluorescently labeled affinity reagents. Immunoaffinity-based electrophoresis typically employs detection of an Ab *via* a direct assay, where fluorescently labeled antigen (Ag*) is in excess as the affinity reagent. The direct assay is based on the reaction:

$$\mathrm{Ab} + \mathrm{Ag}^* \text{ (excess)} \leftrightarrow \mathrm{Ab} - \mathrm{Ag}^* + \mathrm{Ag}^*$$

CE reveals Ab–Ag* and Ag* peaks in which the Ab–Ag* peak is linearly proportional to Ab concentration over specific regions of the dose-response. Ag detection is usually accomplished *via* a competitive assay where Ab is limited in quantity and the fluorescently labeled Ag (Ag*) is the affinity reagent. During incubation, the analyte competes with the Ag* for limited Ab binding sites. The central reaction is thus

$$\mathrm{Ag} + \mathrm{Ag}^* + \mathrm{Ab(limited)} \leftrightarrow \mathrm{Ab{-}Ag} + \mathrm{Ab{-}Ag}^* + \mathrm{Ag} + \mathrm{Ag}^*$$

CE separation resolves Ag* and Ab–Ag* peaks in which the Ag* peak is proportional to increasing Ag concentration over the linear region of the dose–response curve. Ag detection can also be implemented *via* a direct assay using labeled Ab (Ab*) in excess as the affinity reagent. However, small charge-to-mass ratio differences among the Ag–Ab* and Ab* species can make high resolution separations difficult, especially under native conditions [15, 16].

1.2 Lab-on-a-chip integration

While powerful methods, affinity separations face key technical challenges in analysis of clinically relevant analytes. Use of lab-on-a-chip technology can address some of these challenges by offering improved operation of multi-

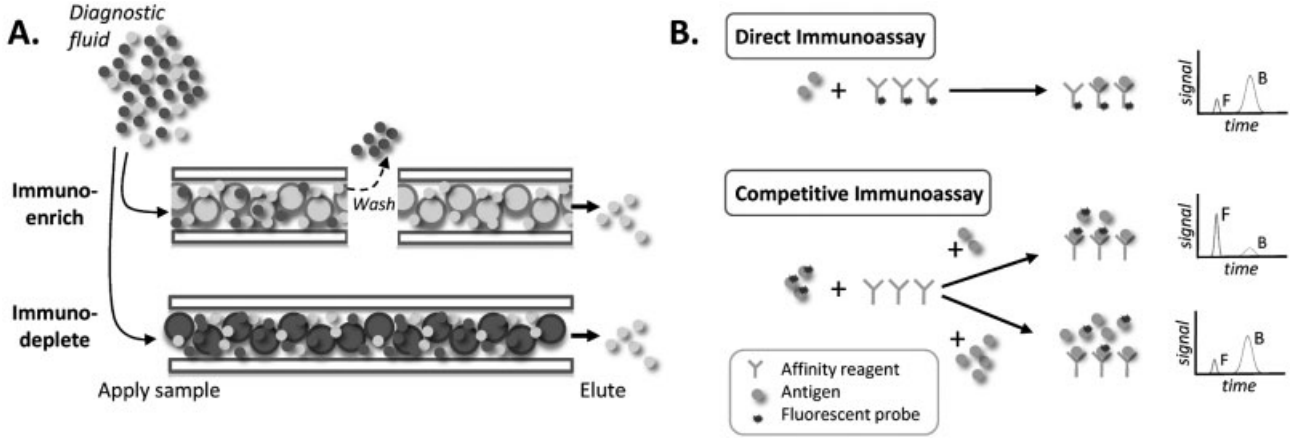

Figure 1. Examples of affinity-based sample preparation and analysis strategies. (A) Immunoenrichment and immunodepletion can be employed to reduce diagnostic fluid complexity. Small symbols indicate serum (dark circles) and target protein (light circles). Large circular symbols indicate beads (or other monoliths) functionalized with affinity reagent specific to target. Beads can be localized on-chip or in capillaries, cartridges, or tubes. (B) Direct and competitive assays can be used to probe for Ag in affinity-based MCE. Schematics at right are idealized electropherograms for each case (B, bound; F, free).

stage assays (*i.e.*, tandem preparatory and analytical functions), substantial analysis throughput, and exceptional assay performance [17]. Microfluidic platforms provide an unrivaled capability to integrate and automate preparatory and analytical functions in a single instrument using a lab-on-a-chip approach [18–20].

A critical challenge for analysis of proteins is analytical sensitivity, as proteins of interest are often present in low abundance [21]. Attaining sufficient analytical sensitivity typically mandates inclusion of sample preparation strategies (see Fig. 1), including immunodepletion and immunoenrichment methods [22]. As in capillary and macroscale bioanalytical methods, incorporation of on-chip protein enrichment and purification steps improves assay lower LOD and accessible dynamic range. On-chip electrokinetically controlled assays with high sensitivity (down to the single molecule level) are beginning to surface in the literature [23]. Consequently, clinical operations are beginning to benefit from microfluidic methods. For such applications, incorporation of multistage assays into a monolithic tool can reduce instrument operator training requirements, making translation of common laboratory operations to near-patient environments potentially feasible.

Integration of multiple time-consuming, labor-intensive steps directly improves throughput. Serial processing of a single sample (*i.e.*, sample preparation followed by analysis) and multiplexed analyses (*i.e.*, analysis of multiple samples and/or analysis of multiple analytes) have both been demonstrated at the proof-of-principle level to benefit from microfluidic technology, as detailed in this review. Lossless, multistage analysis has relevance to low volume diagnostic fluids (*e.g.*, tears, gingival crevicular fluid, and archived samples). In addition to clinical diagnostics, both biomarker discovery and validation benefit from high-throughput [24–26]. With specific relevance to affinity-based electrokinetic analyses, microfluidic technology also benefits assay performance through low sample injection dispersion and favorable heat dissipation during operation (*i.e.*, allowing high operating electric field strengths) [27, 28]. CE and CEC separation performance depends acutely on the maximum separation resolution attainable, as directly impacted by sample dispersion and field strength [27].

This review is divided into sections that chronicle developments in the broad categories of affinity-based electrophoretic and electrochromatographic chip-based assays, as summarized in Table 1. We give additional consideration to advances in assay multiplexing and integrated functionality. We limit our discussion to reports that provide evidence of direct clinical relevance.

2 Microfluidic technology enables high-performance affinity-based separations

To date, small hapten molecules with molecular masses of a few 100 Da to large proteins in excess of 150 000 Da have been analyzed using planar microfluidic geometries. The dominant microfluidic format employed for on-chip affinity-based electrokinetic assays is that of a planar, glass microdevice with double-T sample injectors [27, 29, 30]. Similar devices have been used extensively and successfully for microchip electrophoresis (MCE) and on-chip CEC [31, 32].

2.1 Free-solution affinity-based separations in standard microfluidic geometries

In one of the first microfluidic affinity-based assays, Chiem and Harrison [33] demonstrated a direct assay for monoclonal mouse IgG in mouse ascites fluid. Mouse ascites fluid containing mAb against BSA was incubated off-chip with fluorescein-labeled BSA (BSA*). After a 15 min incubation of sample and probe (BSA*), 10 μL of of the probe-containing solution was analyzed *via* MCE. Separation of the Ab–BSA* complex peak from BSA* was accomplished in under a minute. In the ascites matrix, the peak height of the immunecomplex was linear *versus* anti-BSA concentration over the range of 0–135 μg/mL. The same microchip device was adapted to the analysis of theophylline (Th), a therapeutic drug for asthma, *via* a competitive assay. Human serum samples spiked with Th were first mixed off-chip with fluorescein-labeled theophylline (Th*) and antitheophilline Ab. After a 10-s mixing step *via* bench top vortexing, roughly 10 μL of probe-containing serum was transferred to the chip and analyzed *via* MCE. Free Th* was separated from Ab–Th* complex in under a minute. The Th assay achieved a detection limit of 1.25 ng/mL in diluted serum. A calibration curve of Th* peak area *versus* log(undiluted Th) was linear from 2.5 to 40 μg/mL, covering the important clinical range from 10 to 20 μg/mL for serum. As a means to investigate the reproducibility of the Th serum assay, Chiem *et al.* repeatedly conducted the assay on exogenous Th in serum over a 4-day period and observed an SD in measured Th concentration of 5%. These initial resports suggested a robust, rapid ability to analyze protein content in complex diagnostic fluids using affinity-based MCE.

Using a fused-silica chip, Koutny *et al.* [34] reported on serum cortisol determination over a range of clinical interest (1–60 μg/dL or 30–1700 nM) *via* a competitive assay [34]. Serum sample containing endogenous cortisol was incubated off-chip with fluorescein labeled cortisol and rabbit polyclonal Ab against cortisol. As the majority of cortisol in serum was bound to corticosteroid-binding proteins, a releasing agent (8-anilino-1-naphthalenesulfonic acid) was also added to the serum during incubation. The authors used MCE to resolve a sharp and well-defined peak for free labeled cortisol in less than 30 s. The speed of separation enabled more than 100 separate analyses of a single sample within an hour. Reproducibility of labeled cortisol concentration determination was 1–2% for buffer samples and 3–6% for serum samples. The authors attribute the greater variability observed for serum samples to the presence of salts and confounding proteins in the complex fluid. Rapid and accurate determination of cortisol using affinity-based

Table 1. Microfluidic affinity-based clinical analysis

Reference	Analytes	Clinical relevance	Sample matrix	Microfluidic approach
[34]	Cortisol	Stress indicator; screening for Cushing's syndrome and Addison's disease	Serum	MCE
[33]	Th	Drug analysis	Serum	MCE
[36]	Thyroxine (T4)	Diagnostic of thyroid disorders	Serum	MCE
[37]	Estradiol	Evaluating reproductive functioning	Spiked buffer	MCE with on-chip mixing
[65, 55]	Ins	Long-term monitoring of Type 2 diabetes	Islet secretion	MCE with on-chip islet culture and mixing
[60]	Naproxen	Drug analysis. Monitoring of drug complications and allergic reactions	Plasma	MCE with on-chip mixing
[39]	Tetanus toxin	Measure tetanus Ab and toxin level	Serum	PAGE-MCE
[70]	MMP-8	Diagnostic of periodontal disease	Saliva	PAGE-MCE with on-chip enrichment and mixing
[44]	AFP	Tumor marker; monitor the result of cancer treatment (*e.g.*, chemotherapy)	Spiked buffer	MCE using mobility-shifting DNA-coupled Ab
[45]	AFP	Tumor marker; monitor the result of cancer treatment (*e.g.*, chemotherapy)	Spiked buffer	ITP-MCE using DNA-coupled Ab with on-chip enrichment and mixing
[38]	L3 isoform of AFP	Early identification of hepatocellular carcinoma	Spiked buffer	KCE
[75]	Inflammatory and anti-inflammatory cytokines	Assessment of severity of injury and outcome in patients with brain trauma	CSF	MCE with on-chip immunoaffinity extraction and fluorescence labeling
[77]	Inflammatory biomarkers	Assessment of severity of skin lesion	Solubilized dissected human tissue biopsies	MCE with automated on-chip immunoaffinity extraction and fluorescence labeling
[76]	Inflammatory neuropeptides and post-inflammatory cytokines	Assessment of severity of muscle pain and effectiveness of anti-inflammatory treatment	Tissue fluid	MCE with on-chip immunoaffinity extraction and fluorescence labeling
[52]	IAP	Tumor marker found to have increased concentration in patients' serum	Spiked serum	MCE with on-chip immunoaffinity extraction

MCE has clinical potential in screening of Cushing's syndrome [35], Addison's disease, and in the diagnosis of chronic stress.

As a means to assess thyroid function and thyroid disorders, Schmalzing *et al.* [36] demonstrated a competitive assay for determining total 3,5,3′,5′-tetraiodol-L-thyroxine (T4) in serum using fused-silica microfluidic devices. Undiluted serum was mixed off-chip with fluorescein-labeled T4, T4 releasing agent, and polyclonal Ab. The mixture was subsequently incubated for 30 min at 37 °C. MCE was used to analyze serum samples with T4 levels of 6 and 24 μg/dL, relevant to the adult normal range spanning 5–12 μg/dL in serum. Three replicate electrophoretic analyses were completed within 90 s.

2.2 Affinity-based assay design considerations

With the goal of optimizing affinity-based electrophoretic assay performance, Taylor *et al.* [37] examined the thermodynamic and experimental limitations on detection limits for competitive MCE immunoassays. Based on both theoretical modeling of dose–response curves and experimental results from microchip assays, the authors arrived at the following assay design guidelines: (i) a molar ratio of Ab to labeled analyte of 1:2 maximizes assay dynamic range while optimizing detection limits and (ii) lowering the Ab concentration improves detection limits when the binding affinity of Ab and Ag is high. Using these optimized conditions, Taylor *et al.* developed a competitive assay for estradiol, an important sex hormone and an indicator of reproductive function, using monoclonal anti-estradiol Ab and fluorescein labeled estradiol. The reported affinity constant of the mAb was 2×10^9 M^{-1}. The authors achieved an impressive detection limit of 310 pM (or 85 pg/mL) in spiked buffer, allowing the microfluidic assay to approach the sensitivity of commerical estradiol ELISA kits. As will be discussed later, Bharadwaj *et al.* [38] have reported a model and validating experiments for nonequlibrium lectin affinity-based MCE. The model pre-

dicts electropherograms for a specific assay by incorporating the effects of molecular diffusion, electromigration, nonequilibrium reaction, and the detection process. The authors use the method to optimize their assay and determine kinetic rate constants of the interacting species.

2.3 PAGE for on-chip immunoassays

Herr *et al.* [39] used UV-based photopatterning to fabricate cross-linked polyacrylamide gels in glass microchannels as a means to enhance the resolving power of homogeneous electrophoretic immunoassays. As in conventional slab gels, the cross-linked gels provide a tunable molecular sieving matrix. The cross-linked gels further act to eliminate bulk flow and minimize nonspecific protein adsorption onto microchannel (and capillary) walls. Nonspecific adsorption has been identified as a performance-degrading factor [40], especially as related to separation efficiency and run-to-run reproducibility. Numerous channel coating strategies have been proposed and validated, as summarized by several reviews [41–43]. Sample dispersion arising from bulk flow is eliminated using the cross-linked gels. In the approach reported, a direct electrophoretic assay for tetanus Ab and a competitive assay for tetanus toxin in serum were successfully implemented. A detection limit of 680 pM (or 100 ng/mL) was achieved for tetanus Ab. Clinical applications of the assays include measurement of toxin concentration in serum and evaluation of Ab response after vaccination. Conventional immunoassays such as ELISA can require several hours of assay time. The on-chip native PAGE immunoassays for tetanus Ab and toxin were completed in less than 3 min after an off-chip incubation step.

2.4 Immunoreagent charge heterogeneity suppression using DNA fragments

While direct assays for Ag detection using fluorescently labeled antibodies have a large dynamic range, separation of Ab*–Ag and Ab* is often difficult owing to small charge-to-mass differences and peak broadening inherent in the charge heterogeneity of Ab*. Kawabata and co-workers reported on a direct assay for α-fetoprotein (AFP), a tumor marker of hepatocellular carcinoma and endodermal sinus tumors. The authors utilize the Ag binding antibody fragment (Fab) of the anti-AFP Ab covalently coupled to 626-bp DNA as a means to detect AFP [44]. The DNA-conjugated Fab fragment was incubated off-chip with buffer solution containing AFP for 30 min. Subsequent electrophoretic analysis of the sample was performed using an Agilent 2100 Bioanalyzer. Intercalator dyes were employed for on-chip DNA labeling, allowing sensitive detection of free DNA-conjugated Ab and DNA-conjugated immunecomplex within 90 s of initiating MCE. The width of the 626-bp DNA-coupled Fab fragment peak was ~100 times narrower than that of an Alexa Fluor 647-labeled Fab fragment. The observation supports the conclusion that DNA fragments with high charge-to-mass ratio could sufficiently suppress electrophoretic heterogeneity of an Ab when the two are covalently coupled. The direct MCE assay for AFP achieved a detection limit of ~300 pM (or 21 ng/mL) and a linear concentration response curve up to 20 nM (or 1.4 μg/mL). The group has employed DNA-coupled antibodies as mobility enhancers in subsequent work [45, 46].

2.5 Kinetic affinity-based electrophoresis

Kinetic CE (KCE) refers to electrophoresis of species that interact noncovalently. Noncovalent interactions can be described *via* a reversible reaction: $L + T \leftrightarrow LT$ where L is the ligand and T is the target [47]. As a separation proceeds, the reaction is perturbed away from equilibrium. Applications of KCE include kinetic measurements and affinity-based purification and detection [48, 49]. A recent review by Krylov [47] summarized various implementations of KCE and associated experimental conditions. KCE is particularly useful for separation of isoforms with identical electrophoretic mobility. Recently, Bharadwaj *et al.* [38] implemented KCE in a microfluidic device for detection of *Lens culinaris agglutin* (LCA)-reactive AFP (L3), a specific marker for hepatocellular carcinoma. In the reported implementation, AFP isoforms were first immunoenriched and subsequently analyzed *via* MCE. Discrimination of the L3 isoform from the LCA-nonreactive AFP (L1) isoform was achieved *via* KCE by placing LCA in the running buffer. As the separation proceeded, AFP-L3 interacted with LCA, leading to a mobility shift between the L1 and L3 immunecomplexes. The LCA concentration and applied separation voltage were optimized for the reported system so as to improve peak shapes, separation resolution, and L3 isoform recovery.

2.6 Affinity-based MEKC

MEKC permits the separation of uncharged and charged species through introduction of surfactants (*e.g.*, SDS) above the surfactant CMC in the running buffer. von Heeren *et al.* [50] implemented MEKC on a planar glass microchip housing a cyclic channel network. The cyclic channel design coupled with repeated column switching obtained improved plate numbers for a fixed separation voltage. One of the first competitive immunoassays for serum Th was performed using the microfluidic approach. Serum containing Th was incubated off-chip with fluorescein-labeled Th and Th Ab for 10 min. The addition of SDS containing buffer to the sample mixture led to assay success using uncoated channels. A calibration curve was generated for Th concentrations of 7, 12, and 26 μg/mL in buffer solution. Using the method, the authors reported the serum Th level of a patient in a 50-fold reduced analysis time, as compared to MEKC in a capillary.

2.7 Detection strategies

The aformentioned assays, and the majority detailed in this review, rely on LIF detection. LIF utilizes laser excitation of fluorescently conjugated affinity probes. At the desired separation length, a focused laser beam excites fluorophores present. At the detection point, fluorescence emission is collected through a high numerical aperature objective onto either a PMT or CCD. As fluorescently labeled species migrate past the LIF detector, signal is collected as a function of time yielding an electropherogram. Fluorescent peaks correspond to fluorescently labeled probe, as well as any complexation of probe and target. The height and area of each peak in the electrophoregram can be calibrated to analyte concentration. Using LIF detection, microchip immunoaffinity assays routinely achieve detection limits in the low nM range [33]. Detection limits of affinity-based electrophoretic assays are ultimately limited by the volume of sample reaching the LIF detector. Further improvements in assay sensitivity require sample preconcentration strategies which can be readily integrated on microchips, as mentioned and further described in the following sections.

Alternate detection methods have been explored for affinity-based MCE assays. Detection methods based on chemiluminescence (CL) or electrochemistry do not require excitation sources, thus these nonfluorescence approaches could simplify instrumentation and operation. Mangru and Harrison [51] integrated post separation mixing of CL reagents (*i.e.*, horseradish peroxidase enzyme (HRP), luminol, and peroxide) to perform CL-based detection in microchip affinity-based electrophoresis. The assay relied on the continuous introduction of luminol into the separation channel using electrokinetic flow. Near the end of the separation channel, the device geometry included a microchannel for electrokinetically introducing peroxide prior to detection of the sample peaks. In a direct assay for mouse IgG, sample containing mouse IgG was incubated off-chip with HRP-labeled goat antimouse IgG (HRP·IgG) for 15 min at room temperature. The incubate was placed in the sample well and a sample plug of 60 pL was injected into the separation channel. During the electrophoretic separation, free HRP·IgG separated from IgG–HRP·IgG complex due to differences in electrophoretic mobility. Once HRP labeled compounds reached the end of the separation channel, HRP catalyzed the reaction of luminol and peroxide, resulting in CL. CL signal was captured by a PMT. The assay resolved the free HRP·IgG peak in 30 s. The authors attribute the lack of signal for the IgG–HRP·IgG complex to precipitation of the complex prior to the detection point. Nevertheless, increasing mouse IgG concentration was illustrated by measuring a decreasing free HRP·IgG peak. Using CL detection, the direct assay for IgG achieved a detection limit in the low-nM regime.

In affinity-based CEC assays, bound analytes are routinely retained by affinity reagents immobilized on solid supports and subsequently eluted with low pH buffer for electrokinetic separation. Tsukagoshi *et al.* [52] implemented an immunoassay where the unbound fraction of labeled reagent was transported to the separation channel for CL detection. Glass beads immobilized with Ab specific for target analyte were placed in the sample well of a double T configured microfluidic device. Solutions containing target analyte were mixed off-chip with isoluminol isothiocyanato (ILITC) labeled Ag. Two microliters of incubate were introduced into the sample well for binding with antibodies immobilized on glass beads. Immediately following sample loading, the unbound fraction of the Ag was eluted electrophoretically into the loading channel. At the end of the separation channel, a reaction between the ILITC-labeled analyte with hydrogen peroxide produced CL. The CL signal was captured by a PMT placed underneath the detection point. A competitive assay for immunosuppressive acidic protein (IAP), a cancer biomarker, was performed using the described procedure. The total assay time, including sample loading, separation, and CL detection, was under 2 min. The assay showed linearly increasing CL intensity for increasing IAP concentration in buffer over the range of 100 nM to 5 μM.

Electrochemical detection has also been successfully used for affinity-based MCE assays. Wang *et al.* [53] reported a low detection limit of 2.5×10^{-16} g/mL (1.7×10^{-18} M) in a model assay for mouse IgG using an electrochemical approach. Sample incubation, injection, separation, postseparation enzyme reaction, and amperometric detection were integrated in a microfluidic device. The chip had separate wells containing alkaline phosphatase (ALP)-labeled antimouse IgG (ALP·Ab) and mouse IgG (Ag), which were mixed by alternating electrokinetic injection in a reaction channel before reaching a double-T injector. During MCE, the enzyme-labeled free Ab (ALP·Ab) and the labeled Ag–Ab complex (Ag–ALP·Ab) were resolved. A microchannel near the end of the separation channel was utilized to introduce 4-aminophenyl phosphate (*p*-APP) substrate. When the free Ab and the Ag–Ab complex reached the end of the separation channel, reaction of *p*-APP with the ALP label produced 4-aminophenol (*p*-AP). The liberated *p*-AP was measured amperometrically using on-chip screen-printed carbon electrodes coupled to an off-chip electrochemical detector. The assay time was dominated by the migration time of ALP·Ab and Ag–ALP·Ab in the separation channel. With an applied electric field strength of 256 V/cm, ALP·Ab and Ab–E·Ag reached the detector 125 s and 340 s after injection, respectively. Electrochemical detection holds promise for decentralized clinical or environmental testing.

3 Monolithic devices enable multiplexed assay formats

Multiplexed assays have been reported in various microfluidic implementations. Assays can be found implemented as: parallel analysis of a single sample for multiple analytes

using analyte-indexed channels, parallel analysis of multiple samples for a single analyte using sample-index channels, a hybrid of these two analysis approaches, and analysis with concurrent calibration separations using calibration-specific and sample-specific microfluidic channels. While recent reports detail significant advances in said multiplexed formats, work regarding multiplexed affinity-based electrokinetic assays using lab-on-a-chip technologies is still in its infancy.

3.1 Parallel channel networks for concurrent affinity-based assays

Cheng *et al.* [54] reported on devices capable of independently performing six concurrent assays, each equipped with on-chip mixing and electrophoretic separation capability [54]. Two additional channels were reserved to house dyes for optical alignment during LIF scanning detection. Data were acquired using a single-point fluorescence detector with a galvano-scanner to step between separation channels. The authors demonstrated simultaneous direct immunoassays for ovalbumin and for anti-estradiol using MCE. The on-chip mixing, reaction, and separation steps were performed within 60 s in all cases and within 30 s using optimized conditions, including simultaneous calibration runs.

Dishinger and Kennedy [55] developed a chip containing four individual channel networks, each capable of performing immunoaffinity-based electrophoretic analysis of perfusate from insulin (Ins)-producing cells found in the pancreas known as islets of Langerhans. The islets were housed in a small chamber and continuously perfused with biological media. EOF was used to sample perfusate-containing secreted Ins into a 4 cm long reaction channel. Each channel network allowed on-line mixing of perfusates with FITC-Ins and anti-Ins Ab for competitive detection of Ins secretion. The reaction streams were sampled at 6.25 s intervals and analyzed in parallel using an on-chip CE separation with LIF detection by a scanning confocal microscope. The LOD for Ins was 10 nM. The affinity MCE approach was capable of completing over 1450 assays of islet secretion in less than 40 min.

For both multiplexed assays described, the authors noted that the electrical interface for voltage control poses, potentially, a limitation on the number of parallel assays performed on a single chip. One example of a solution to the electrical interfacing challenge was reported by Bromberg and Mathies [56], wherein the authors arranged 48 channel networks in a radial pattern for affinity-based electrophoretic detection of trinitrotoluene (TNT). An electrode ring array was placed in the sample wells for simultaneous injection, which greatly reduced complexity of the electrical interface, while a laser scanning detector efficiently generated electropherograms for multiple channels [54, 55].

3.2 Optimized affinity reagents for multiplexed analyte detection in a single channel

Simultaneous detection of multiple biomarkers in a single biological sample is advantageous for increasing throughput. However, analyte multiplexing in a single channel is relatively hard to achieve in free solution electrophoresis when using an Ab as affinity reagent. The separation difficulty arises from minute differences in charge-to-mass ratio among immunecomplexes (Ag–Ab) [57]. To facilitate single-channel, multianalyte assays, Kawabata *et al.* [44] used DNA fragments having various base pair lengths to act as mobility modulators when coupled to antibodies to AFP and prostate-specific Ag (PSA). The high charge-to-mass ratio of the DNA fragments allowed appreciable separation among different Ag–Ab complexes. The authors demonstrate simultaneous detection of AFP and PSA in buffer solution using a 626-bp DNA-coupled anti-AFP Ab and a 245-bp DNA-coupled anti-PSA Ab. As shown in Fig. 2, peaks 4 and 7 correspond to the single Ab conjugate complex for PSA and AFP whereas peak 8 corresponds to a double Ab sandwich complex for AFP. The immunecomplexes were resolved from internal standards and unbound DNA-coupled antibodies in less than 90 s.

4 Toward integration of sample preparation and affinity-based electrokinetic assays

Active development efforts center on improving and extending the functionality of affinity MCE. In particular, several groups are reporting notable advances achieved through incorporation of technology and strategies not common in capillary-based systems.

4.1 On-chip mixing and incubation of reagents and sample in mixing channels

While immunoaffinity MCE allows rapid analysis (<5 min), the total assay time has often been dominated by the off-chip sample incubation steps. Incubation of sample with labeled probe has been reported as taking anywhere from several minutes up to an hour at room temperature. In efforts to reduce total assay time, several groups have devised schemes to implement on-chip sample incubation. In an early work, Chiem and Harrison [58] implemented a competitive assay in which on-chip mixing of diluted serum samples with labeled tracer and Ab was integrated with separation and analysis. The authors simultaneously load diluted serum samples containing Th and Th* into a 26.5 mm long mixing channel. Subsequently, the Th and Th* containing sample was mixed with anti-Th Ab in an 81.6 mm long serpentine channel to allow formation of Ab–Th and Ab–Th* complexes. The 52 μm wide mixing channels allowed a uniform transverse concentration of the small Th molecule in ~4 s.

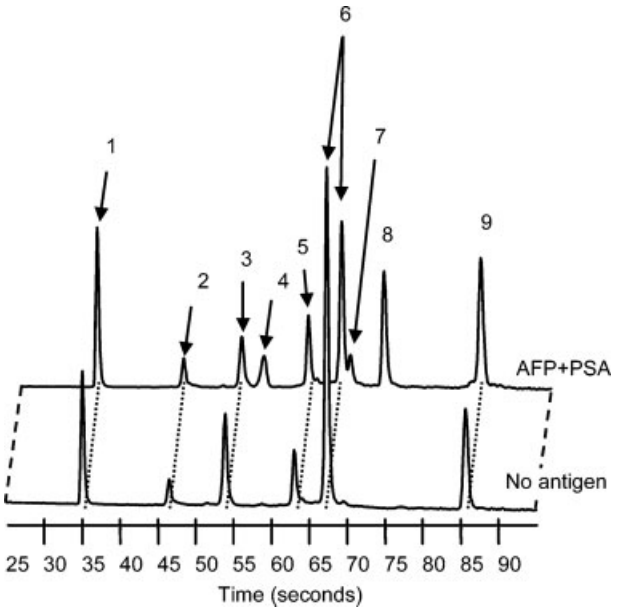

Figure 2. Electropherograms from affinity-based MCE show simultaneous detection of PSA and AFP using mobility-modified mAbs. The PSA mAb was conjugated to 245-bp DNA, while the mAb to AFP was conjugated to 626-bp DNA. Reprinted from [44], with permission.

Under the reported flow conditions, the time required to achieve a uniform transverse concentration multiplied by migration time allowed the authors to determine the required length of each mixing channel. The device was first operated in a continuous-flow mode to give a 32 s immunoreaction time in the mixing channels, followed by separation and detection completed within 1 min. The device was also operated in a stopped-flow mixing mode to further increase the incubation time. The authors found that the competitive immunoaffinity MCE was nearly completed in ~1.5 min, as opposed to 15 min using an off-chip incubation. The reported competitive Th assay achieved a detection limit of 1.3 ng/mL, which was consistent with the detection limit determined for off-chip mixing followed by on-chip separation [33].

In later work, Qiu and Harrison [59] used mixing channels similar to those of Chiem *et al.* for on-chip generation of calibration curves *via* electrokinetic solvent proportioning. Calibration curves were generated by running an assay multiple times, each serial analysis having a different known analyte concentration. In an assay to directly detect anti-BSA Ab with fluorescently labeled BSA, electrokinetic flow was used to proportionally mix sample containing anti-BSA with a diluting buffer in a 26.5 mm long mixing channel. The approach provided a variable anti-BSA concentration for downstream mixing with BSA*. Mixing ratios of anti-BSA (Ab) and the diluting buffer were controlled by varying the voltage applied at the Ab well and at the diluting buffer well. Starting with an initial anti-BSA concentration of 46.8 μg/mL, the electrokinetic solvent proportioning scheme allowed the authors to program an anti-BSA concentration calibration from 3.9 to 43.1 μg/mL. A linear calibration curve was generated for anti-BSA concentrations ranging from 7.7 to 39.3 μg/mL. This scheme of on-board generation of calibration curves by electrokinetic solvent demonstrates an auto-calibrating functionality, potentially quite useful for high-throughput applications as well as clinical diagnostics.

Rapid assessment of nonsteroidal anti-inflammatory medications drugs such as naproxen has clinical relevance in diagnosing potential drug complications and side effects ranging from gastric ulceration to severe allergic reactions. Phillips and Wellner [60] utilized MCE for rapid measurements of naproxen in human plasma. In a direct naproxen assay, plasma and AlexaFluor 633 Ab* were introduced into a mixing channel *via* EOF for 2 min incubation reaction. A serpentine separation channel was found to increase the efficiency of MCE to resolve Ab*–Ag complex from free Ab*, especially for a small molecules such as naproxen. With a 110 mm separation distance to detector, the immunecomplex was detected by LIF in 100 s and the excess Ab was resolved in a further 30 s. The total assay time was 5 min. The device achieved a detection limit of 25 ng/mL in both buffer and plasma samples with saturation level greater than 450 μg/mL. MCE of spiked plasma samples correlated well with that obtained using conventional HPLC, but was more than three times faster. The microchip was applied to measuring naproxen concentration in plasma sample from different patient groups. For the patients tolerating the treatment, the microchip measured naproxen concentrations of 78–137 μg/mL in plasma. For the patients with allergic reactions, the microchip measured naproxen concentrations ranging from 205 to 364 μg/mL. The authors observed that the increasing naproxen concentration correlated to the severity of allergic reaction.

4.2 On-chip proteolysis for peptide analysis

In a hybrid microfluidic-capillary system, Yue *et al.* [61] utilized a microfluidic format for sample preparation (proteolysis), while performing electrophoretic analysis using conventional capillary-based instruments (CE and capillary LC/MS). To accomplish the on-chip proteolysis, the authors packed agarose beads containing immobilized trypsin in a microchannel. Following proteolysis, digested peptides were pressure-flushed through a channel packed with agarose beads immobilized with ferric ions. Phosphorylated peptides bound to the functionalized beads. Using β-casein as a target analyte, the device showed selective enrichment of two expected phosphopeptide fragments in CE-based separation and detection. The authors attribute detection of four additional phosphorylated fragments to incomplete proteolysis.

Slentz *et al.* [62] reported on integration of protein digestion, affinity-based selection, and CEC using a PDMS microchip. As shown in Fig. 3, microfabricated frits divided a microfluidic channel into several compartments. Silica sor-

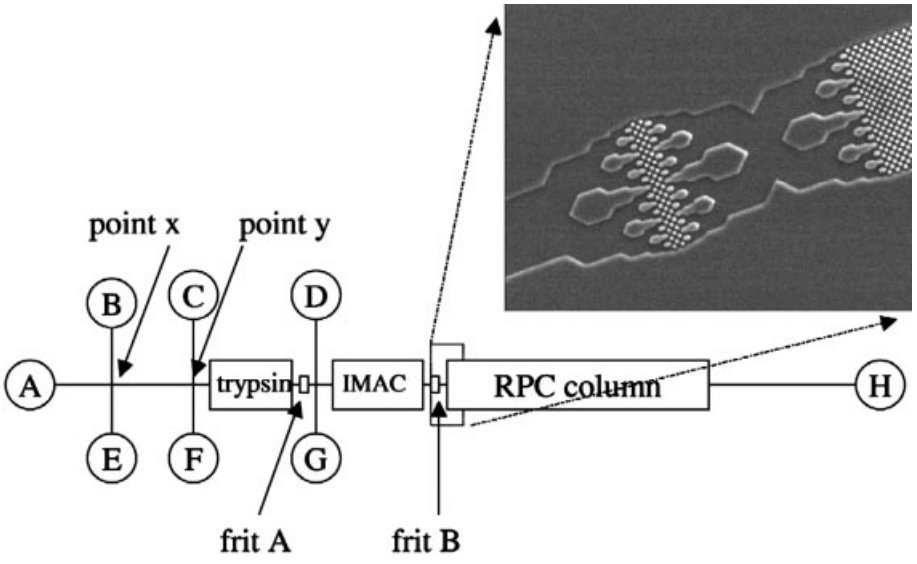

Figure 3. Single chip integration of trypsin digestion, copper (II)-immobilized metal affinity chromatography (Cu(II)-IMAC) selection of histidine-containing peptides, and RP CEC of selected peptides. Reprinted from [62, with permission.

bents modified with trypsin for proteolysis were loaded into a first compartment *via* EOF. Cu(II) immobilized sorbents for histidine-selection were then loaded into a second compartment. A third compartment contained a co-located monolithic support structure for peptide fractionation. In a model demonstration, FITC-BSA was transported through the trypsin digestion bed and eluted directly to the Cu(II)-loaded column where histidine-containing peptides were retained. Histidine-containing peptides were later eluted off of the Cu(II)-loaded column using EDTA. An electrophoretic separation followed by fluorescence detection on the monolith support structure resolved individual histidine-containing peptides in 2 min. On-chip metal affinity selection and separation of the FITC-BSA digest were consistent with results obtained on a conventional HPLC system. However, on-chip BSA digestion was observed to be less complete than that performed in a standard packed column. The authors utilized on-chip proteolysis followed by affinity selection and detection as a means to study protein structure and activity.

4.3 On-chip immunoenrichment and depletion integrated with electrophoresis

Dodge *et al*. implemented a heterogeneous electrochromatographic assay for rabbit IgG. The authors employed rabbit IgG-specific protein A immobilized on glass microchannel walls as a means to exploit the high surface area-to-volume ratio of microchannels for high-efficiency affinity interactions [63]. Pressure-driven flow patterning of a silanizing reagent was used to pattern the linker chemistry for subsequent protein A attachment. Electrokinetic pumping was utilized to implement a direct assay for Cy5-IgG. Sample was first pumped continuously over the immobilized protein A to allow binding of Cy5-IgG with protein A for 30–300 s. After sample incubation, excess sample was washed away with Tris-HCl buffer (pH 7.5) and bound sample was eluted off the affinity-capture region using glycine–HCl buffer (pH 2.0). Subsequent direct detection was performed on the eluted sample using LIF. The dissociation of rabbit IgG from protein A was instantaneous and irreversible, therefore concentrating the rabbit IgG into a narrow zone. The Cy5-IgG assay required less than 5 min to complete. The reported detection limit of 50 nM approaches the clinical detection limit for IgG targeted by the authors. A competitive assay for rabbit IgG was also performed by incubating rabbit IgG and fluorescently labeled rabbit IgG with protein A either simultaneously or in a sequential manner. The serial implementation was found to reduce required sample incubation times ten-fold (from 300 s to 30 s). The authors note that the automated rabbit IgG heterogeneous assay can be extended to other analytes by binding a primary Ab to the protein A functionalized surfaces.

4.4 Sample preparation and lectin affinity chromatography

Glycosylated protein isoforms (also known as glycoforms) can be probed with lectins, which are multivalent proteins not of immune origin. Lectin affinity chromatography is a powerful technique that can separate protein glycoforms into fractions based on differential affinity of a protein and associated glycoforms toward specific lectins. Mao *et al*. [64] adapted lectin affinity chromatography to a microfluidic chip, which reduced total analysis time from 4 h to less than 7 min. The *Pisum sativum agglutin* lectin has affinity specificity toward glycans containing terminal mannosyl residue or an *N*-acetylchitobiose-linked fucose residue. Mao *et al*. utilized this lectin to distinguish among different glycoforms of various egg white glycoproteins (turkey ovalbumin, chicken ovalbumin, ovomucoid). In the reported imple-

mentation, a 500 μm long porous monolith was fabricated within a microfluidic channel by UV photopatterning. Immobilization of *P. sativum agglutinin* was accomplished through the interaction of epoxy groups in the polymer monolith and the ε-amino groups of the lectin. Using EOF, egg white glycoproteins were driven through the *P. sativum agglutinin* impregnated monolith allowing *P. sativum agglutinin* to interact with various glycans. Glycoforms with no affinity toward the lectin were first washed away with HEPES buffer (pH 7.49) and detected downstream by LIF. Weakly adsorbed glycoforms were then eluted using a solution containing a low concentration displacing sugar. Finally, a solution containing a high concentration displacing sugar was used to elute strongly adsorbed glycoforms. All washing steps were automated using a voltage sequencing program that greatly simplified the separation process as compared to the macroscale implementation. In addition, only 300 pg of glycoprotein was required for the analysis.

4.5 Integrated cell culture and affinity-based electrophoresis of secreted proteins

To perform on-line monitoring of Ins secretion from islet cells, Roper *et al.* [65] designed microfluidic devices allowing mixing of effluent from an islet with FITC-labeled insulin (Ins*) and anti-Ins Ab in a 4 cm long reaction channel *via* EOF. The mixing time was controlled by adjusting the applied electric potential. For formation of Ab–Ins* complexes comparable with off-chip mixing, on-chip mixing required 50 s and was augmented by thin film heaters that raised the temperature in the mixing chamber to 38°C, as a means to produce favorable binding kinetics. Following mixing in the reaction channel, samples were electrokinetically injected onto a 1.5 cm long electrophoresis channel where the Ins* and Ab–Ins* complex were separated in 5 s. The integrated on-chip mixing and fast electrophoretic separations allowed Ins measurements at 15 s intervals, thus enabling continuous monitoring of Ins secretion with detection limits of 3 nM (or 18 ng/mL). The method was reported to resolve secretory profiles of both first- and second-phase Ins secretions upon addition of glucose to the islets. The work highlights use of microchip immunoaffinity assays for high temporal resolution monitoring of cellular secretions of soluble analytes.

4.6 Protein enrichment and mixing at gel membranes integrated with affinity-based separations

On-chip incubation and mixing methods that rely on coflowing species in mixing microchannels are efficient for small analyte molecules that quickly diffuse across typical microchannel geometries. Assays for larger molecules can require appreciably longer mixing channel lengths than those required for small analytes. Long mixing lengths can lead to an undesirable increase in the required chip area. While in-chip photopatterned size exclusion membranes and structures have been used to preconcentrate proteins before separation and detection [66–69], such structures also act as efficient reactors for on-chip sample incubation and mixing. Herr *et al.* [70] integrated a small pore-size polyacrylamide gel membrane contiguous with a separation gel as a means to enrich low-abundance proteins and provide efficient mixing of sample with immunoreagents. During device operation, fluorescently labeled antimatrix metalloproteinase-8 Ab (αMMP-8*) was electrophoretically loaded against the membrane. Diluted human saliva sample was then loaded against the membrane. For saliva containing the MMP-8 enzyme, an MMP-8 immunecomplex formed. Confining analytes and antibodies to a small volume at the membrane interface resulted in exceptionally efficient mixing and rapid incubation. Simply increasing the duration of preconcentration can enhance assay sensitivity and dynamic range [71]. No differences between assays performed with on-chip mixing and assays with 15 min off-chip incubation were observed. The species were electrophoretically eluted off the membrane and analyzed by native PAGE, which resolved MMP-8 complex from excess αMMP-8* in <2 min. The technique developed by Herr and co-workers achieved a detection limit of 130 ng/mL for MMP-8 protein, which is significantly lower than MMP-8 concentration in saliva of periodontally diseased patients (623.8 ± 204 ng/mL). As shown in Fig. 4a, analysis of endogenous MMP-8 in a retrospective pilot patient cohort compared well with measurements made using gold-standard ELISA assays. Additionally, microchip measurement of endogenous MMP-8 was able to discriminate among patients in different stages of periodontal disease as shown in Fig. 4b. The technology was developed for use in a clinical setting, here forming the basis of a diagnostic capable of assessing MMP-inhibitor therapy in treatment of periodontal disease.

4.7 ITP as an enrichment strategy for affinity-based electrophoresis

Automated sample preconcentration and stacking has also been achieved with transient ITP [72, 73]. Mohamadi *et al.* [74] demonstrated an ITP-assisted electrophoretic immunoassay for detection with anti-HSA Ab using labeled HSA (HSA*). Sample incubated with fluorescent probe was injected into the analysis channel, sandwiched between two different buffer solutions: a leading buffer with Cl^- ions and a terminating buffer with low mobility Gly^- ions. During analysis the sample plug was captured between the terminating and leading buffers, resulting in transient sample stacking and enrichment in less than 1.5 s by transient ITP. A size-based separation in methylcellulose solution resolved HSA* from Ab–HSA* in 25 s within a 1 cm separation length. The ITP-based sample stacking increased the HSA* and Ab–HSA* peak intensities by 40- and 270-fold, respectively. Increasing the Cl^- concentration in the leading buffer can further enhance the fluorescence intensity at the expense of reducing migration time differences between

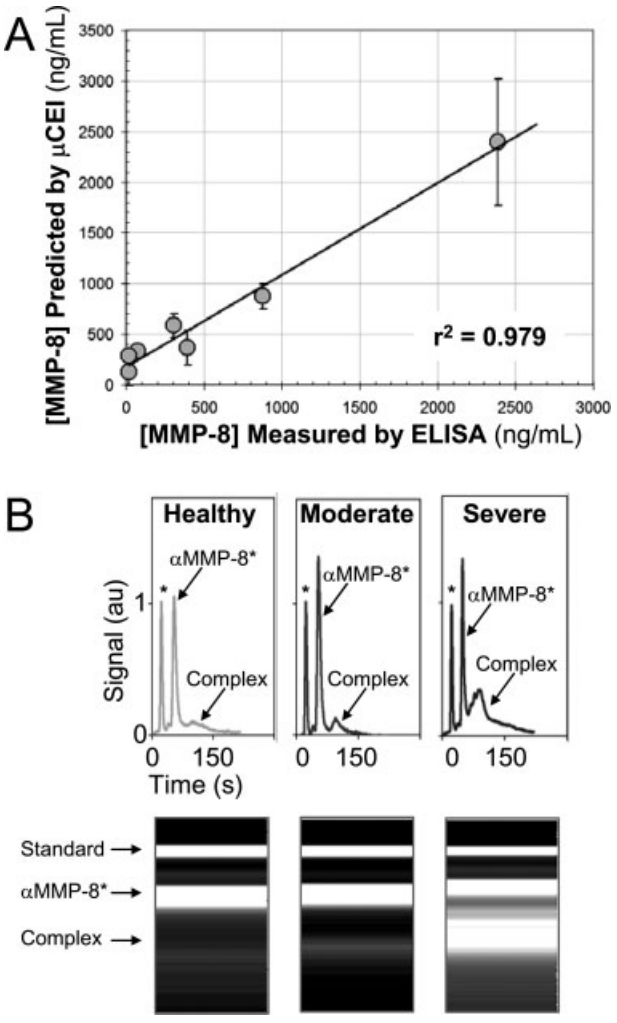

Figure 4. Integrated protein enrichment and mixing with MCE immunoassays allows measurement of endogenous MMP-8 in saliva. (A) Linear regression analysis for the MMP-8 concentrations measured by MCE immunoassays and commercial ELISA. (B) MCE immunoassays for MMP-8 measurements from a healthy patient and patients clinically diagnosed as having moderate to severe periodontal disease reveal differences in MMP-8 levels. Reprinted from [70], with permission.

adjacent peaks. This direct assay for labeled HSA achieved a detection limit of 7.5 pM, a remarkable improvement compared to conventional homogeneous immunoassays performed using MCE.

Recently, Park *et al.* [46] combined ITP for on-chip sample preconcentration and stacking with gel electrophoresis in a microchip assay for direct detection of AFP using DNA coupled anti-AFP Ab. Diluted human serum spiked with AFP and DNA coupled anti-AFP Ab solution were injected simultaneously into a mixing channel for on-chip sample incubation. The analyte to Ab ratio was modified by changing applied loading voltages. In the ITP mode, the sample was stacked using a high conductivity leading buffer upstream and low-conductivity trailing buffer downstream. The stacking factor was calculated to be $\sim 200\times$. Once the sample migrated downstream into the separation channel, the applied voltage was switched to gel electrophoresis mode in which the sample was surrounded by mainly the high-conductivity leading buffer. Free DNA coupled Ab and DNA coupled Ab–Ag complex were separated and detected within 90 s. Data quality of the electropherograms such as peak intensity and peak resolution were affected by how much low conductivity trailing buffer entered the separation channel, which was determined by the delay time between the ITP and gel electrophoresis mode. Even varying the delay time from 0 to 1 s could significantly increase signal intensity while decreasing peak resolution. The delay time could be precisely controlled by computer programmed voltage sequencing, thus optimizing data quality and reproducibility.

In a recent publication, Kawabata *et al.* [45] observed that concentrating Ab using ITP prior to in-channel immune reaction resulted in 140-fold increased signal over directly concentrating off-chip incubated immunecomplex. The authors developed an "electrokinetic analyte transport assay" integrating mixing, reaction, and separation on-chip. In a direct assay for AFP, the previously mentioned tumor marker, two anti-AFP mouse mAbs (clones WA1 and WA2) recognizing different AFP epitopes were utilized to form a sandwich immunecomplex. After loading sample, the respective antibodies, trailing buffer, and leading buffer to individual wells on the microchip, pressure driven flow was applied to establish five zones in the main electrophoresis channel: trailing buffer (containing HEPES ions), first Ab (245-bp DNA conjugated WA1 Ab, DNA·WA1), sample (containing AFP), second Ab (HiLyte dye-labeled WA2 Ab), and leading buffer zone (containing Cl^- ions). Upon application of an electrical potential, DNA·WA1 migrated into the sample zone and reacted with AFP while it was stacked and concentrated by ITP. Consequently, the AFP-DNA·WA1 immunecomplex migrated into the second Ab zone by ITP, forming the sandwich immunecomplex consisting of DNA·WA1, AFP, HiLyte·WA2. ITP stacked the immunecomplex into a narrow zone. Gel electrophoresis further resolved the fluorescently labeled sandwich immunecomplex from nonspecific fluorescent material. The total assay time was 136 s, with an ITP time of 63 s and gel electrophoresis time of 73 s. The device achieved a detection limit of 5 pM for AFP in leading buffer and a linear concentration response curve up to 630 pM.

4.8 Immunoenrichment integrated with electrophoresis for analysis of multiple cytokines

Phillips [75] implemented a CE system with immunoaffinity capture for rapid measurement of six inflammatory cytokines in cerebral spinal fluid (CSF) of patients suffering from head trauma. The immunoaffinity capture module was constructed by immobilizing six mAbs against the target cytokines on the sample port of a double-T configured CE

device. A minimal ratio of Ab to analyte of 15:1 was required to efficiently capture analytes in clinical CSF samples. During analysis, 500 nL of CSF sample was first introduced to the sample port and incubated for 5 min to allow binding of target cytokines with immobilized antibodies. Following incubation, the sample was removed from the port using a syringe. A solution of AlexaFluor 633 dye was subsequently introduced into the port for *in situ* labeling of bound analyte. After 5 min of incubation, the dye solution was removed and the port was washed with buffer. Finally, low-pH phosphate buffer was introduced into the port to dissociate bound analyte from immobilized antibodies. Released cytokines including interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α, interleukin-10, and transforming growth factor-β were analyzed by MCE, where individual cytokines were resolved in 2 min. Using LIF detection, the device achieved a detection limit of 0.5–1.6 pg/mL for cytokines in buffer and 0.6–2.1 pg/mL for cytokines in CSF. The approach was used to analyze cytokine concentrations in CSF from patients with various degrees of head trauma. The study revealed elevated levels of inflammatory cytokines associated with increasing extent of injury, as well as showing correlation of the analytes with prognosis. Furthermore, the MCE determined CSF cytokine levels compared favorably to levels determined with ELISA (R^2 value >0.95).

A similar device and approach was used to quantify 12 different inflammation-associated mediators in tissue fluid samples from patients suffering from muscle pain [76]. The immunoaffinity capture module was constructed by immobilizing mAbs against 12 target mediators in the sample port of a double-T configured MCE device. Here, a minimal ratio of Ab to analyte of 50:1 was required to efficiently capture analytes. Following incubation and on-chip fluorescence labeling, the inflammatory mediators substance P, calcitonin gene-related peptide, brain derived neurotrophic factor, vasoactive intestinal peptide, neuropeptide Y, neurotrophic factor-4, β-endorphin, adreno-corticotropic hormone, corticotrophin releasing hormone, interleukin-1β, interleukin-6, and tumor necrosis factor-α were resolved by MCE in 160 s. Using LIF detection, the device achieved a detection limit of 0.6–2.0 pg/mL in buffer solution. The microchip was used to analyze inflammatory mediator concentration in tissue fluid from healthy volunteers and patients suffering from mild to intense muscle pain. Microchip measurement revealed elevated levels of neuropeptides and proinflammatory cytokines for the patient group treated with intramuscular injections of the anti-inflammatory agent cortisol. MCE also validated the anti-inflammatory effect of cortisol as tissue samples obtained 30 min postcortisol treatment showed dramatically lower concentrations of inflammation-associated markers. Finally, MCE assessed the kinetics of mediator release using samples collected every 5 min post-treatment from the affected muscle.

In a later work, Phillips and Wellner [77] developed a microdevice to automate sample incubation, affinity-based analyte isolation, laser dye labeling, and analyte elution with pressure-driven and electrokinetic flow. The device was utilized to quantify 12 inflammatory biomarkers from human skin biopsies. Solubilized dissected tissue was introduced to the sample port *via* a pump and allowed to incubate for 3 min with the panel of 12 mAbs immobilized in the sample well in a fashion similar to their previous reports. Following incubation, nonbound materials in the sample well were flushed to waste *via* pressure-driven flow. A solution containing a fluorophore was electrokinetically injected into the sample well for on-chip fluorescent labeling of captured biomarkers. After a 2 min on-chip labeling reaction, low pH elution buffer was electrokinetically introduced into the sample well thereby releasing the bound analytes. The immunoenriched biomarkers interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α, interferon γ, transforming growth factor-β, macrophage inflammatory protein-1α, macrophage chemoattractant protein 1, substance P, calcitonin gene related peptide, neuropeptide Y, and vasoactive intestinal peptide were analyzed using MCE. Using LIF detection, all 12 biomarkers were resolved in 2.2 min. Owing to automated fluid handling, a complete analytical cycle took just 9 min. The approach achieved a detection limit of 1.85–6.55 pg/mL for cytokines in buffer solution. MCE measurements of the 12 inflammatory biomarkers were able to distinguish among skin biopsies obtained from patients with inflammatory skin lesions and samples from healthy volunteers. The assay also showed decreasing inflammatory biomarker concentration in tissue areas 5 and 15 mm away from a lesion site. As shown in Fig. 5, patients with chronic lesions showed elevated levels of cytokines, as compared to patients with minor lesions.

5 Conclusions

The nascent transformation of clinical medicine to personalized medicine would be bolstered by sophisticated diagnostic tools available at the point-of-care [78]. Especially relevant for assays on minimally processed diagnostic fluids (as is preferred for point-of-care use [9]), microfluidic technologies foster instrument design that incorporates multistage preparative and analytical functions. While a budding endeavor utlizing lab-on-a-chip technology, clinical assays that effectively handle preparation of samples as diverse as plasma, serum, urine, tissues, and proximal fluids are needed for specific disease classes. Further, clinical diagnostics that rely on single-marker approaches assume that a change in the concentration of a single protein or analyte can unambiguously specify disease. Diseases exhibit substantial heterogeneity between individuals; the same disease can be initiated by numerous factors and cause a range of molecular changes and physical manifestations. Consequently, many state-of-the-art single marker diagnostics suffer from a lack of clinical sensitivity and specificity [21]. In contrast, diagnostics that measure or "profile" multiple protein bio-

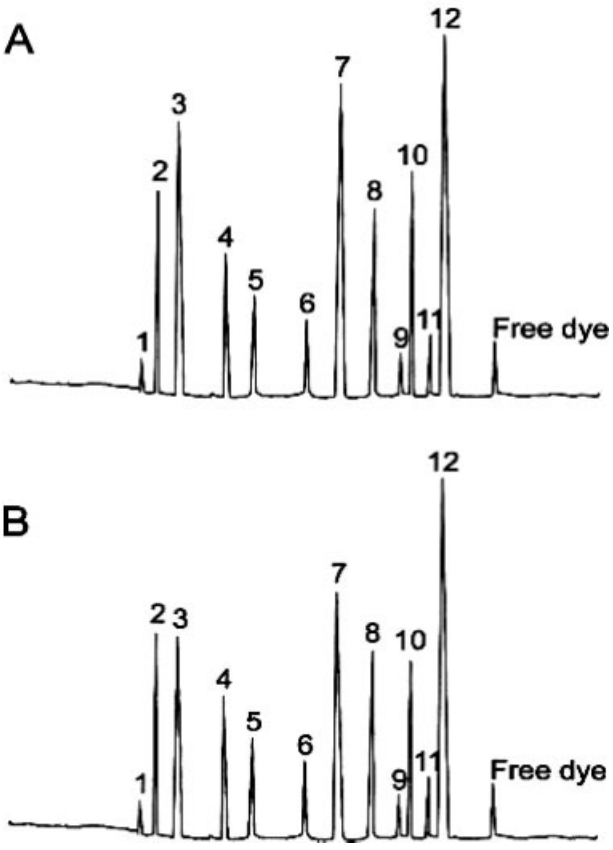

Figure 5. Multiplexed analysis of 12 inflammatory biomarkers from tissue biopsies. (A) Typical electropherogram of cytokine concentrations recovered from a patient with chronic lesions. (B) Typical electropherogram of cytokine concentrations recovered from a patient with mild lesions. Peaks: 1, TGF-b; 2, IL-6; 3, IL-1b; 4, IFNg; 5, MIP-1a; 6, MCP-1; 7, TNF-a; 8, CGRP; 9, NY; 10, IL-8; 11, VIP; 12, SP. Reprinted from [77], with permission.

markers may perform more effectively. We see the development of multiplexed (multianalyte) clinical and point-of-care diagnostic tools benefitting from microfluidic integration and rapid analyses.

While progress in microfluidically enabled diagnostics is at the cusp of making a positive impact on diagnostics for screening, monitoring and efficacy assessment, substantial efforts focused on validation of putative protein disease biomarkers are sorely needed. Dismal success in translation of protein disease biomarkers to the diagnostic arena has emerged as a perplexing development of the last decade [21]. In spite of significant advances in proteomic technology, few new protein biomarkers have emerged from the proteomic discovery pool, progressed though the scrutiny of validation studies, and become incorporated in diagnostic tools [25, 26]. In a compelling analysis of this difficult biomarker pipeline problem, Zolg boldly posits that the biomedical community has a tendency to overrate the biomarker discovery phase and under-appreciate another challenge facing personalized medicine in the 21st century: the arduous task of developing and undertaking rigorous, candid assessments of biomarker candidates within carefully planned validation schemes [24, 25]. Key technology specifications include providing measurement reproducibility and reducing the required labor and time necessary to complete a large-scale validation study. Without a concerted, cohesive effort to develop the instrumental infrastructure required for high-throughput, reproducible validation studies, the critical gap between biomarker discovery and translation of said biomarkers to point-of-care diagnostic tools remains [79].

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