OPHTHALMOLOGIST-ON-A-CHIP: FULLY INTEGRATED MICROFLUIDIC TEAR OSMOLARITY AND PROTEIN BIOMARKER QUANTIFICATION FOR DRY EYE STRATIFICATION

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ABSTRACT

Human tear film fluid offers tremendous potential as a non-invasive diagnostic fluid. However, the use of tear fluid in diagnostic medicine is limited owing to non-standardized collection methodologies and low volume availability. We present a first-reported microfluidic tear fluid biomarker assay which will serve as a foundation for both rapid biomarker validation and point-of-care ocular diagnostics. Our microfluidic approach demonstrates multiplexed quantitation of both tear fluid osmolality and lactoferrin protein concentration in microliter quantities of human fluid from healthy and Sjögren’s syndrome (SS) patients.

KEYWORDS: Lactoferrin, Tear Film Fluid, Electrophoresis, Immunoassay

INTRODUCTION

Human tear film fluid is composed of a complex mixture of proteins, glycoproteins, lipids, and small molecules. Importantly, several eye-specific tear biomolecules have been shown to be putative biomarkers for diseases ranging from autoimmune diseases (e.g., Sjogren’s syndrome) to ocular bacterial infections rampant in the developing world (e.g. trachoma) [1]. As a result, human tear fluid has significant potential as a non-invasive diagnostic fluid [2]. Despite the promise it offers, the use of tear fluid in ophthalmic medicine is limited.

Major technological hurdles hinder routine clinical assessment of tear fluid biomolecules. These gaps include: low throughput (> 5 hours for analysis for ELISA), lack of automation potential, consumption of large sample volumes, and limited sensitivity levels for clinical assessment. Taken together, these gaps make current assays (i.e., sizing, ELISA, osmometry) unsuitable for point-of-care diagnostics and rapid quantification of endogenous protein or osmolality levels in clinically relevant ranges. Consequently, rapid, quantitative, highly specific assays for endogenous tear proteins and tear osmolality would impact a spectrum of needs in the ophthalmology clinic: from the ability to quantitatively assess dry eye disease severity to the ability to stratify mechanistically-distinct patient populations for targeted treatment.

In particular, a rapid biomarker-based assay to assess dry eye severity and stratify Sjögren’s syndrome (SS) patients from other forms of dry eye disease would provide tremendous clinical benefit. SS is a severe form of keratoconjunctivitis sicca (KCS or dry eye disorder) and is a systemic autoimmune disease that is estimated to impact 1-2% of the general population [3]. Early diagnosis is important for timely disease management and the prevention of permanent tissue damage. However, currently, diagnosis requires an invasive salivary gland biopsy, making the average time to diagnosis over 6 years [3].

Tear osmolality stands out as a biomarker to assess dry eye severity as it has been shown to increase in KCS patients and is thought to be directly proportional to disease severity [4]. Further, lactoferrin (Lf), is a high-abundance tear-specific protein which is down-regulated in the tears of SS patients when compared with non-SS KCS patients [5]. Importantly, the development of a biomarker-based assay for the differential diagnosis of SS and severe KCS would eliminate the need for salivary gland biopsy and promote timely treatment of glandular and extraglandular manifestations of SS.

This paper presents important advances in a first-in-kind multiplexed microfluidic assay for tear fluid biomarkers of dry eye disease. Importantly, while the focus of the present study is measurement of osmolality and Lf, other important protein markers of disease are amenable to measurement using the approach. To our knowledge, no similar efforts exist. The following sections present theory and device design followed by fabrication technique, experimental results and finally the relevant discussion and conclusions.

THEORY

Microfluidic tools are uniquely suited to rapidly quantify tear osmolality and endogeneous proteins in volume-limited tear samples. As detailed in Figure 1, our approach integrates on-chip electrical impedance measurements for osmolality quantitation with an electrophoretic immunoassay for Lf. The assay takes advantage of the high specificity of antibody-antigen interactions to separate fluorescently labeled free antibody from bound antibody-antigen immune complex for a quantitative measure of antigen concentration in a complex sample. Immunoassays offers numerous advantages over current techniques including rapid species resolution (seconds), high analytical specificity, low sample volume requirements (nL to µL), the potential for facile multiplexing to assess multiple proteins in a single sample, and a small form factor for point-of-care, automated use.

A polyacrylamide gel was used within the channels as shown previously [6] and the gel pore-size was tuned in order to minimize dispersion of the immune complex band. Photo-patterned discontinuous gel architectures in particular have recent-
ly been shown by our group to enhance sensitivity levels and allow for ultra-short separation distances, opening the possibility for point-of-care, battery powered devices that employ analytical grade electrophoresis [6]. To enable specific quantitation of Lf, an alkaline protein (isoelectric point of 8.7), the buffer conditions were optimized to pH 11 using an N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (pKa 10.5). These high pH conditions minimize positive residues on the surface of the protein that are available to non-specifically bind to other factors in the tear matrix as well as assay substrates.

Figure 1 presents a schematic overview of the final assay concept. Key design features include: (i) well-characterized sample collection and processing for meaningful assay read-out, (ii) electrophoretically-driven assay steps for hands-free automation, and (iii) high pH buffer conditions to minimize nonspecific matrix effects that are common in tear fluid analysis.

**EXPERIMENTAL**

**Fabrication.** A 3-6%T discontinuous polyacrylamide gel architecture was fabricated as described previously [6]. Fabrication conditions were optimized to separate fluorescently-labeled anti-human Lf antibody (Ab*) and Lf immune complex.

**Assay operation.** The assay steps as detailed in Figure 1 are as follows: 1) collect the tear fluid from the eye surface using a Schirmer strip, 2) reconstitute the tear proteins by incubating the Schirmer strip in PBS, 3) introduce Ab*, 4) pipette the sample onto the microfluidic chip well, 5) electrophoretically load the sample on-chip, 6) once the same is fully loaded (indicated by steady-state measured current), measure the conductivity of the sample to assess sample osmolality, 7) apply orthogonal electric field to inject a small sample plug into the separation channel, 8) allow the free antibody and bound immune complex peaks to resolve from each other based on their differential mobilities, and 9) detect the intensity profile using inverted epifluorescence microscopy and a CCD camera.

**RESULTS AND DISCUSSION**

Using this new approach, we have demonstrated the ability to detect and quantify tear osmolality and endogenous Lf in < 1 µL of healthy and SS tears. Figure 2A shows the quantitation capabilities of the on-chip osmolality measurement using an osmolality standard in CAPS buffer. A 3P curve fit with the formula $y = \beta_1 x / (\beta_2 + x) + \beta_3$ was used to relate measured current and osmolality ($\beta_1 = 6.33$, $\beta_2 = 229.07$, $\beta_3 = 4.91$). A quantitative dose response curve for osmolality is achieved within a clinically relevant range for KCS diagnostics. Figure 2B presents the quantitative capabilities of the homogeneous immunoassay for tear Lf. To provide an accurate quantitative measure of Lf in solution, increasing amounts of Lf were spiked into diluted SS tears and separated on-chip. Complex peak areas were extracted from the fluorescence images and normalized by a BSA internal standard. A nonlinear least-squares fit with a 4-parameter logistic fit model with the formula $y = 0.7 + (0.5 - 0.7)/(1+(x/262.4)^{0.4})$ was used to establish the relationship between Lf concentration and normalized complex peak area ($\beta_1 = -0.5 \text{nM}$, $\beta_2 = 0.7 \text{nM}$, $\beta_3 = 262.4 \text{nM}$, $\beta_4 = 0.4$).

![Figure 1](image1.png)

*Figure 1: Human tear fluid osmolarity and biomarker quantitation is integrated in a reusable, automated platform.*

![Figure 2](image2.png)

*Figure 2: Tear osmolality and Lf quantitation is possible in clinically relevant ranges and < 1 µL of tear fluid. This enables the clinical stratification of two autoimmune disorders (SS & KCS) at the point of care.*
The microfluidic assay measures increased tear osmolality in SS patients compared to healthy controls, consistent with literature (Figure 3A). The on-chip osmolality measurement was validated through comparison to gold standard AC bipolar conductivity meter and Wescor vapor pressure osmometer (Figure 3A). The on-chip method is accurate to within 18% of the osmometer. Further, although the on-chip method under-predicts the tear osmolality in both healthy and SS patients, it is a more accurate predictor of osmolality than using the AC bipolar conductivity meter.

The homogeneous immunoassay measures decreased tear Lf SS patients compared to healthy volunteers, consistent with literature (Figure 3B). On-chip Lf measurements in patient samples agree well with gold-standard ELISA ($r^2 = 0.9941$) and the assay is accurate to within 15% of ELISA with a lower limit of detection of 3 ± 2 nM Lf.

Assay specificity is also crucial for a diagnostic measurement. Especially due to the complexity and relatively unknown composition of tear fluid, efforts must be made to control for any non-specific interaction of anti-human Lf Ab* with other components of tears. Samples of Ab* in Lf-depleted tear fluid were separated on-chip and compared to separations of Ab* and purified Lf spiked into the same Lf-depleted tear matrix. The detectable complex peak areas were compared and demonstrate < 15% nonspecific signal and a high degree of assay specificity for Lf in healthy and SS tears (Figure 3C).

CONCLUSION

For the first time, we demonstrate technologies critically needed for non-invasive tear-based diagnostics. Namely, we report on a multiplexed microfluidic assay for osmolality and Lf protein biomarker quantitation in human tears. Importantly, the microfluidic assay requires minutes to complete and microliter sample volumes, providing the foundation for first-in-kind, highly specific, rapid clinical diagnostics relevant to ocular and systemic diseases.

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