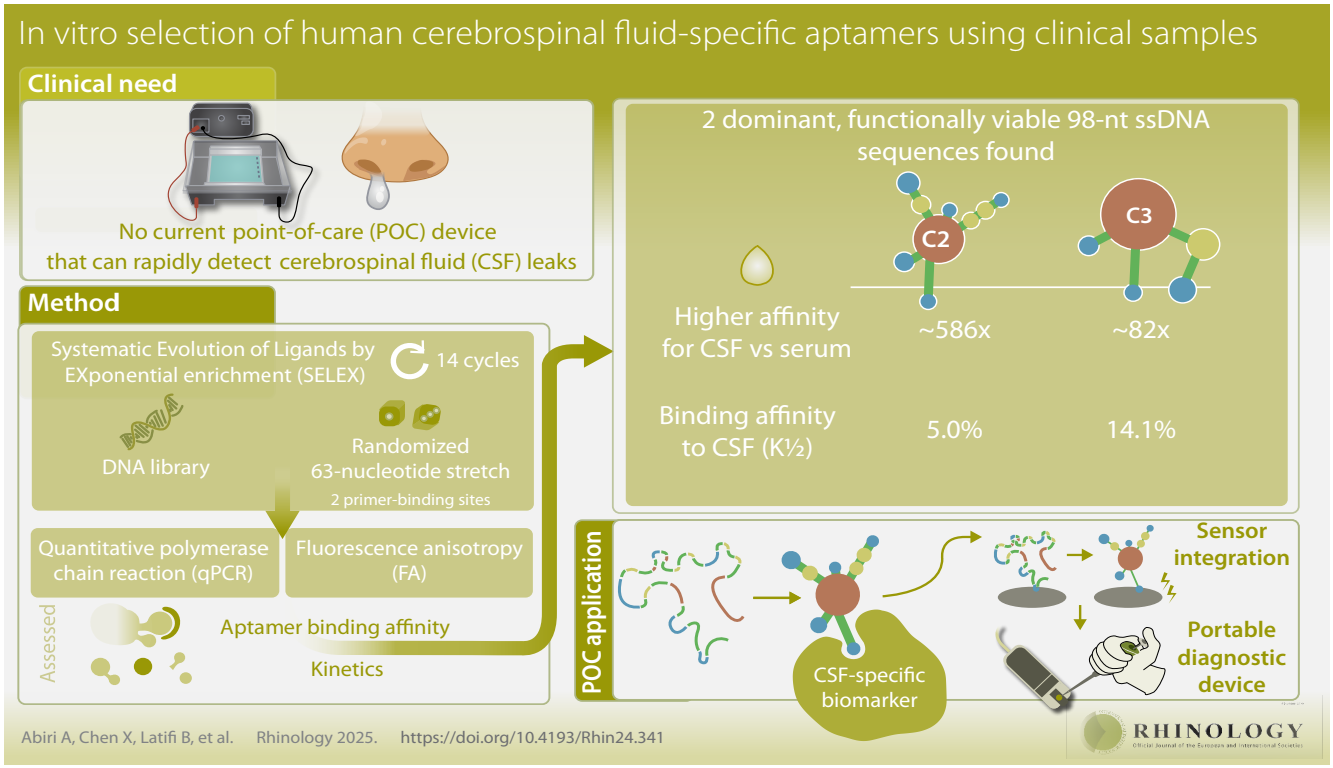
In vitro selection of human cerebrospinal fluid-specific aptamers using clinical samples

Arash Abiri[1,2,*], Xinlei Chen[1,*], Brandon Latifi[3], Frank P.K. Hsu[4], Andrej Lupták[3], Michelle Khine[1], Edward C. Kuan[2,4,#]

Rhinology 63: 1, 103 - 112, 2025
https://doi.org/10.4193/Rhin24.341



Abstract

Background: Cerebrospinal fluid (CSF) leaks may occur due to numerous etiologies and are associated with severe morbidity. Currently in the U.S., confirming the presence of a CSF leak requires protein electrophoresis testing, oftentimes involving specialized processing, and there exists no point-of-care (POC) device for CSF detection. We aimed to discover a single-stranded deoxyribonucleic acid (ssDNA) aptamer capable of selectively binding to CSF-specific biomarkers, with the future goal of developing an aptamer-based POC CSF detection device.

Methods: To identify a candidate aptamer, we performed Systematic Evolution of Ligands by EXponential enrichment (SELEX) using a DNA library containing a randomized 63-nucleotide (nt) stretch flanked by 2 primer-binding sites. Quantitative polymerase chain reaction (qPCR) and fluorescence anisotropy (FA) assessed aptamer binding affinity and kinetics.

Results: Following 14 SELEX cycles, 2 dominant and functionally viable 98-nt ssDNA sequences (C2 and C3) were found. C2 and C3 demonstrated ~586x and ~82x higher affinity for CSF compared to serum, respectively. Increases in FA upon aptamer exposure to higher CSF concentrations demonstrated a $K_{1/2}$ of 5.0% and 14.1% for C2 and C3, respectively.

Conclusions: In vitro selection of a diverse pool of ssDNA sequences yielded 2 aptamers with high selectivity for CSF-specific biomarkers, with potential for integration into a rapid POC electrochemical diagnostic system.

Key words: aptamer, cerebrospinal fluid, CSF leak, point of care device, SELEX

Introduction

Cerebrospinal fluid (CSF) leak, which may occur anywhere along the skull base and emerge from the ear or nose, can be a result of a defect in the closed central nervous system due to trauma, surgical complication, intracranial pressure (ICP) elevation, or may be surgically created during skull base surgery [1–3]. Untreated CSF leak can lead to life-threatening consequences including meningitis, intracranial infection, or death [4–6], warranting prompt treatment that may range from conservative measures to urgent surgical treatment [3]. Therefore, prompt and accurate diagnosis of CSF leaks is critical to delivering high-quality patient care.

Unfortunately, distinguishing CSF leaks from physiologic nasal/otic secretions can be challenging, as patients commonly exhibit increased secretions after trauma or surgery [3,7–9]. Furthermore, certain leaks can have subtle or delayed presentations, making diagnosis challenging [10]. For most clinicians, characterizing a colorless and odorless discharge such as CSF involves a high degree of clinical suspicion and may include a combination of history and physical examination, imaging, and laboratory testing. Imaging modalities largely play a role in localization, but not confirmation [11]. Moreover, signs and symptoms of CSF leak are nonspecific, thereby risking misdiagnosis even by the most experienced of clinicians [7,12]. Conversely, exploration of a suspected CSF leak is more definitive, but if negative, is associated with medical and surgical risks and, for postoperative cases, possible disruption of an intact repair or worsening pneumocephalus, as well as increased healthcare costs.

In the U.S., the current gold standard technique to diagnose suspected CSF leaks involves beta-2 transferrin (β2TF) immunofixation [13–16]. Transferrin, a glycoprotein essential for iron homeostasis, is present in multiple forms within the human body. β2TF is one such isoform in the brain and constitutes approximately one-third of the total transferrin in CSF; it is created as a result of neuraminidase activity that eliminates terminal sialic acid residues on the transferrin glycan chain [17]. Among various biochemical assays used for detecting CSF, β2TF is regarded as the most reliable and specific biomarker [18–20]. Its absence in physiologic nasal/otic secretions or blood makes it a favorable biomarker, hence its use in various techniques such as gel electrophoresis, immunofixation, or isoelectric focusing for detecting CSF with as high as 100% sensitivity and 94% specificity [13–16], though false negative results are certainly possible [21,22]. Current state-of-the-art technology allows for accurate β2TF detection via electrophoresis; however, this technique's accessibility and practicality has been largely hindered by its latency time (i.e., 3-5 days for centers without in-house capabilities) and need for sample purity, adequate sample quantity, and skilled professionals at a specialized laboratory [14,16,23]. As a send-out test at many laboratories and centers, capabilities to test for β2TF may not be readily available in many lower resource areas (i.e., rural centers), which can impact care.

For this reason, other noninvasive quantitative methods that involve measuring glucose, total protein, or prealbumin concentrations in nasal drainage have since been developed to serve as alternatives for diagnosing CSF leaks. However, these tests have been found to be nonspecific and are not recommended for guiding medical management [24]. Recently, Bradbury et al. published their experience developing a point-of-care (POC) CSF detection device on the basis of quantifying CSF-specific protein concentration. However, this technique relied on extensive sample pre-processing by a skilled technician, signaling via liquid color change, had a relatively high limit of detection (~1 mg/L), and demonstrated ambiguous results in samples contaminated by blood [25,26]. Additionally, the aforementioned device utilized beta trace protein (BTP), which, though commonly used in Europe, is also present in the serum, heart, and testes, and thus may be prone to false positive results [27]. Other attempts at developing POC technologies have utilized novel, though non-standard and unvalidated, molecular targets [28], and/or have not been tested in the setting of contaminated samples [29,30].

Electrochemical sensors are recognized for their high sensitivity, selectivity, and rapid response time. They detect trace amounts of substances, differentiate between compounds, and offer real-time data for swift reactions to changes. Their portability, low power consumption, and long-term stability make them ideal for medical diagnostics. Electrochemical aptamer-based (EAB) sensors offer a unique advantage due to the specificity and sensitivity of aptamers. Aptamers are single-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) molecules that bind to specific target molecules with high affinity, akin to antibodies. Aptamers are identified through their highly selective interaction with proteins using an approach known as Systematic Evolution of Ligands by EXponential enrichment (SELEX) or in vitro selection – starting with a target protein with known function, a large and diverse random sequence DNA library is screened for specific interaction [31–33]. When integrated into electrochemical sensors, aptamers enhance the device's selectivity, allowing for precise detection of target molecules. This specificity, combined with the electrochemical sensing mechanism, results in sensors with rapid response times and exceptional sensitivity, capable of detecting trace amounts of the target molecule.

To date, there exists no aptamer specific to CSF-specific biomarkers. With the goal of developing an EAB sensor for POC CSF detection, we sought to discover a novel aptamer with high binding affinity and specificity for CSF-specific biomarkers. Specifically, we aimed to develop a conformation-switching aptamer, which would not only bind to its target, but also change conformation upon binding. This is a desirable property for integration with an EAB sensor since, when exposed to the target molecule, the aptamer can undergo a conformational change ("switch") that can be coupled with a measurable change

in conductance. Thus, in this study, we implemented in vitro selection of DNA conformation-switching aptamers to recognize biomarkers in CSF.

Materials and methods

Materials

Streptavidin magnetic beads (Pierce, Appleton, WI, USA) and 10X DPBS were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Amicon ultra centrifugal filter (50 kDa), phenol solution, Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The DNA library (Polyacrylamide Gel Electrophoresis [PAGE] purification), MB primer (High Performance Liquid Chromotagraphy [HPLC] purification), and the biotinylated capture probes were purchased through Integrated DNA Technologies (IDT; Coralville, IA, USA).

Sequence design

We designed a pool of DNA sequences containing a 63-nucleotide (nt) stretch of random sequences flanked by two primer-binding sites. This ssDNA pool was further modified to contain a molecule of MB at its 5′ terminus. The primers used to amplify the pool by PCR and to make single-stranded variants of it also contained 5′ MB, so that in all rounds of the in vitro selection the ssDNA from which the CSF biomarker-binding aptamers were to be identified, all contained MB at the 5′ termini. We also designed a "capture oligonucleotide" (a biotinylated DNA oligo) that base-paired to the first 10 nts at the 5′-terminus of the pool. This was a key element of the selection, as the capture oligonucleotide was bound to streptavidin beads and the pool was introduced to facilitate binding of the ssDNA pool to the capture oligonucleotide. The 10 base-pair interaction was strong enough to capture the pool at ambient temperatures, but weak enough that a conformational change that disrupted even 2 or 3 of the interacting nucleotides would result in release of the sequence from the beads. All sequences are available in the Supporting Information.

Immobilization of capture probes and DNA library

10 µL of streptavidin magnetic beads was washed three times with 200 µL of 1X DPBS with 0.1% of Triton X-100, and resuspended with 1 µM of biotinylated capture probe. After 5-min incubation using gentle rotation, the liquid was removed, and beads were resuspended in 60 µL of DPBS.

To generate the ssDNA library, the MB primer was used to amplify the starting DNA library using a final concentration of 1X Taq Reaction Buffer, 0.2 mM dNTPs each, 2 µM MB primer, and 1.25 U of DNA Taq Polymerase. The reaction was amplified for 10 to 16 cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s) to determine the optimal amplification. A 3% agarose gel electrophoresis was used to visualize the amplification product for each round.

The MB modified library (2 µL) was hybridized with the capture probe on the magnetic beads by incubating for 5 minutes with gentle rotation. The liquid was removed and beads with the hybridized library were resuspended in 200 µL of 1X DPBS for selection.

In vitro selection of CSF aptamers

The magnetic beads with the hybridized library were washed twice with 200 µL of 1X DPBS prior to the counter selection. For the first to fifth rounds, a final concentration of 1% pooled human plasma (Innovative Research, Los Angeles, CA, USA) with 2 mM EDTA (Thermo Fisher, Waltham, MA, USA) and 1X DPBS was added into the beads as a counter selection. From the sixth to ninth rounds, 1% pooled human serum (Innovative Research, Novi, MI, USA) with 1X DPBS was used as the counter selection and from the tenth to the thirteen round, 50% serum was used as the counter. Finally, the fourteenth round of selection included 100% serum as counter. Serum elutions from rounds 10, 13, and 14 were collected for next-generation sequencing (NGS) analysis. After the counter selection step, a wash with 1X DPBS was performed prior to eluting DNA in the presence of CSF. CSF was then added to the beads and incubated for 5 minutes using gentle rotation to elute DNA from the magnetic beads. CSF fractions were collected and a phenol-chloroform extraction was performed to separate ssDNA prior to precipitation with 300 mM KCl, 1 µl Glyco-Blue and 2.5 volumes of cold 100% ethanol at -20°C. The ssDNA was further purified using a 50 kDa Amicon filter to remove any water soluble protein in the sample, and again precipitated as described above.

The purified ssDNA was polymerase chain reaction (PCR) amplified prior to the next round. The PCR reaction consisted of 1X Standard Taq Reaction Buffer, 0.2 mM dNTPs each, 1 µM of both the forward and reverse primers, ssDNA, and 1.25U Taq polymerase. The ssDNA was amplifed similar to the described method above except the annealing temperature was set to 65°C for 30 seconds. A 3% agarose gel electrophoresis was used to check the amplified DNA prior to the beginning of each selection round.

Illumina sequencing and processing

The CSF elution from rounds 7-10, 13, and 14, as well as the countered serum elutions from rounds 10, 13, and 14 were prepared for Illumina MiSeq sequencing (San Diego, CA, USA). DNA was amplified using the TruSeq forward and reverse adapter in a final reaction containing 1X Standard Taq Reaction Buffer, 0.2 mM of dNTPs, 1 µM of each primer, and 1.25 U DNA Taq polymerase. The reactions were amplified for one cycle (95°C for 30 s, 65°C for 30 s, 72°C for 30 s, follow by multiple repeated cycles (95°C for 30 s, 70°C for 30 s, and 72°C for 30 s) until desired products were visible. Primer amplification was confirmed using a 3% gel electrophoresis prior to purifying DNA with a Zymo

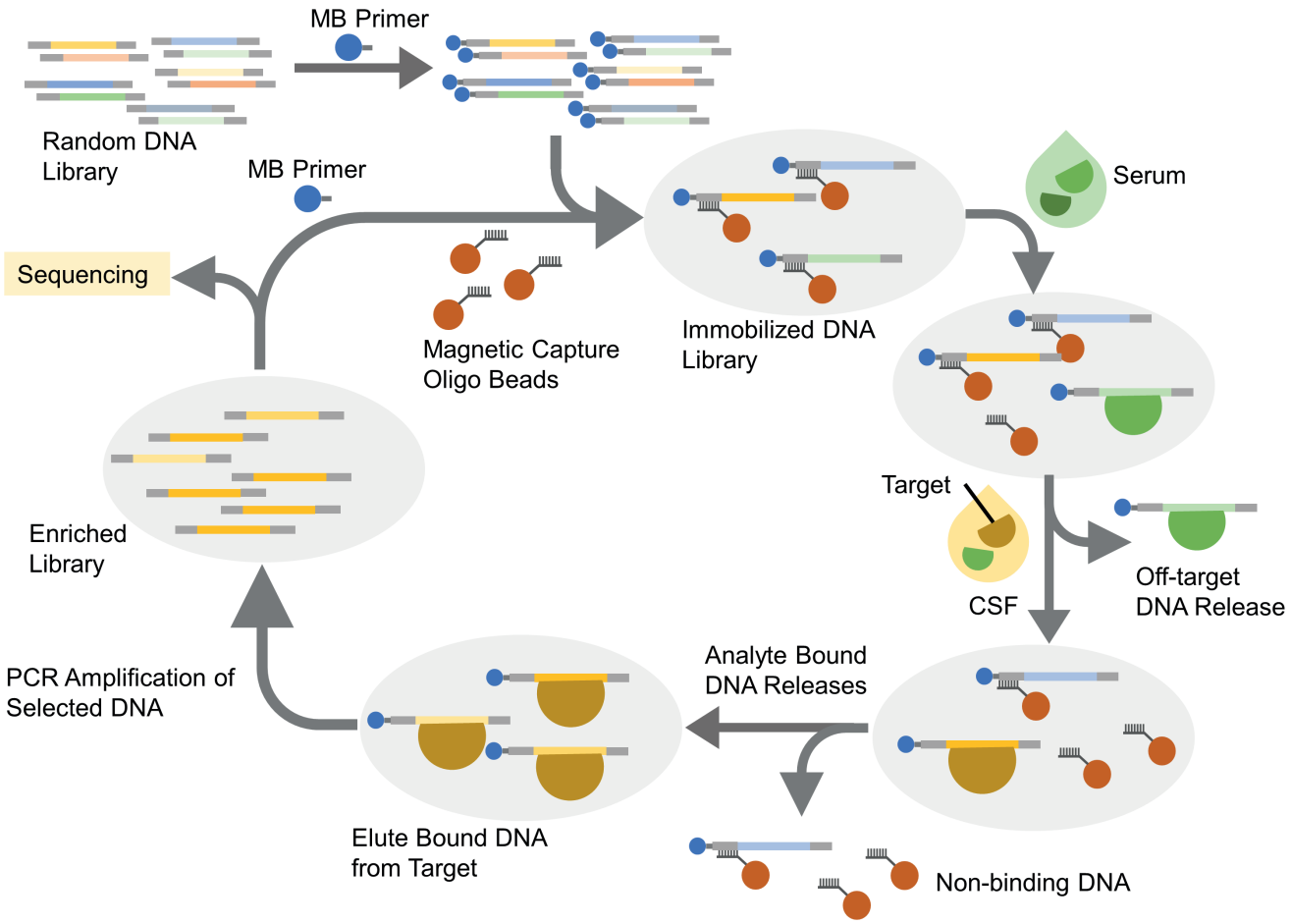


Figure 1. In vitro selection of structure-switching aptamers. A random-sequence DNA library modified with methylene blue (MB, blue circle) at the 5′ terminus was immobilized on magnetic beads (orange circle) via biotinylated capture oligo. Serum was used to wash off ssDNA with low specificity for CSF biomarkers (i.e., binders of non-CSF proteins). Upon adding CSF, the CSF-specific ssDNA underwent a biomarker-dependent conformational change, binding to the target and "switching off" from the beads. These ssDNA unbound from the beads were isolated and amplified to become the new library for the next round of selection.

DNA Gel Extraction kit (Tustin, CA, USA). DNA was then submitted to NGS.

Sequenced reads were obtained using an Illumina MiSeq. Afterwards, reads were merged with Paired-End Read Merger (PEAR) using default settings and Bowtie2 was used to remove any reads matching the PhiX genome [34,35]. FASTAptamer was used to count and then cluster sequences with an edit distance of 10 [36]. Candidate aptamers were identified from the enrichment of sequences throughout multiple selection rounds and then characterized for binding to CSF.

Testing DNA aptamers against CSF

The DNA constructs and the MB primer were used to amplify the ssDNA aptamer for hybridization to the capture oligonucleotide. The beads were washed with 1X DPBS with 0.1% Trition-X-100 and all washes were collected for further analysis. After, 7 M UREA with 40 mM EDTA was added to the streptavidin beads and the suspension was heated at 95°C for 2 minutes to remove any remaining DNA. After, DNA from all six washes were phenol-chloroform extracted and purified using a 50 kDa Amicon filter. Quantitative PCR (qPCR) was used to assess the amount of DNA that was present within each fraction. All qPCR reactions were performed with a final concentration of 1X iTaq Universal SYBR Green Super mix (Bio-Rad laboratories, Hercules, CA, USA), 1 µM forward and reserve primers. The amplification reactions were performed on a CFX Connect Real-Time PCR (Bio-Rad). Cq values were subsequently determined for each fraction and a standard curve was used to calculate the amount of DNA in each sample.

Binding affinity of CSF aptamers

Fluorescence anisotropy (FA), wherein plane-polarized light is used to excite a fluorophore and the extent of depolarization of emitted fluorescence from rotational diffusion is measured, was used to characterize aptamer-target binding. When bound to

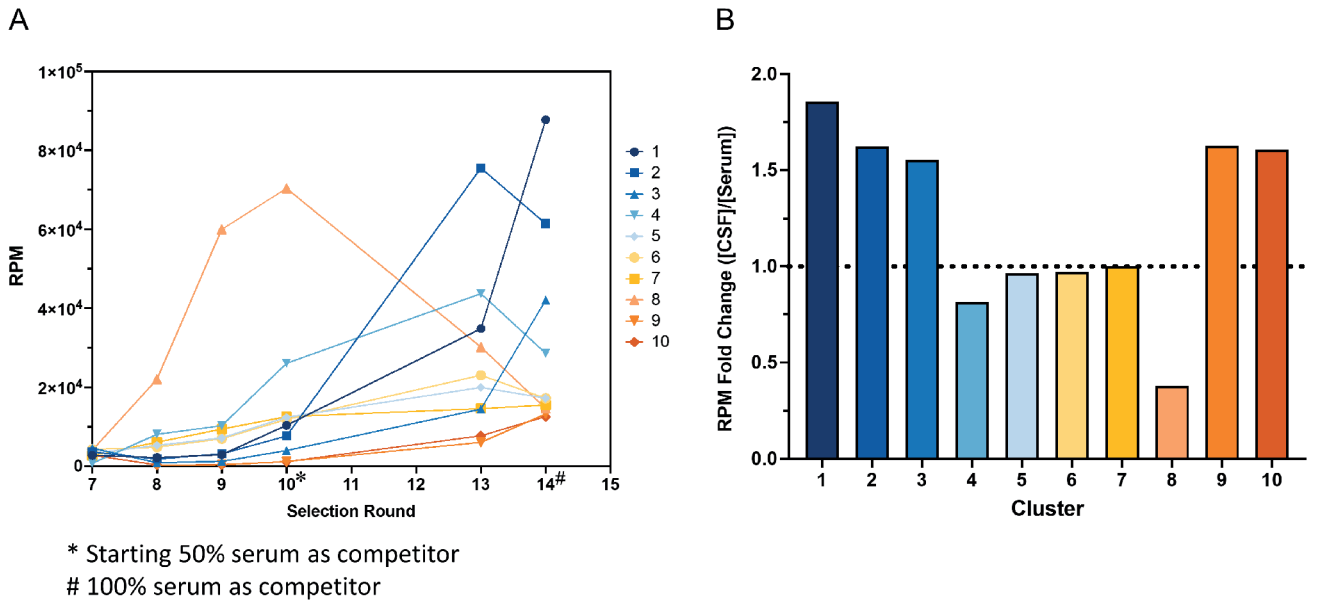


Figure 2. Next generation sequencing analysis of the ssDNA population. (A) Evolution of RPM counts of the top 10 most abundant clusters from selection rounds 7-14. The top 10 clusters were based on sequencing data from round 14. (B) RPM ratio of CSF wash to serum wash on the top 10 clusters from cycle 14. The black dotted line indicates an RPM ratio of 1. A ratio greater than 1 indicated that the cluster sequence was more abundant in the CSF wash than in the serum wash.

its target, the aptamer is expected to undergo slower rotational diffusion and emit greater depolarized light, resulting in higher anisotropy. FA was performed using aptamers containing a Cy3 on the 5' end. Each individual construct was amplified with a Cy3 primer using the same amplification protocol from the selection. The FA experiment was performed using a spectrofluorometer (JASCO FP-6300, Jasco Inc., Easton, MD). Excitation and emission wavelengths used for Cy3 were 545 and 565 nm, respectively. Fluorescent intensities were measured with the combination of the Cy3 ssDNA amplicon and diluted CSF in the final percentages of 1%, 3%, 5%, 10%, 20%, 40%, 60%, and 87%. Since CSF possesses a similar viscosity to water, FA measurements were unlikely to have been confounded by a change in sample viscosity at different CSF concentrations [37]. Three data points were collected for all anisotropy measurements and the averaged numbers were used for data processing. The anisotropy *r* of Cy3 labeled DNA was calculated based on the following equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad where\ G = \frac{I_{HV}}{I_{HH}}$$

Where *I* represented the fluorescence intensity and the subscripts represented the orientation of the polarizers at the emission and detection channel, respectively. Apparent $K_{1/2}$ was determined by fitting the data to the Langmuir Isotherm equation using non-linear (weighted) least-squares regression in R (version 3.6.1; R Foundation for Statistical Computing).

Size exclusion binding assay via qPCR

Magnetic beads (30 µL) were washed and immobilized with capture oligonucleotides following the immobilization protocol. Single sequence of the MB amplicon was hybridized to the beads following the protocol described above. Then the beads were washed twice in DPBS before aliquoted into 3 portions. 200µL CSF sample was centrifuged using 100kDa Amico ultra centrifuge filter. Both the filtrate solution and the concentrated colute were collected and transferred to separate tubes. DPBS was added to each tube to bring the volume back to 200uL. Two fractionated CSF and whole CSF were added into each portion of the beads. Beads were incubated for 10 minutes in gentle rotation before the diluted CSF was obtained for further extraction and purification. qPCR was performed following the protocol described above.

Secondary structure prediction

The secondary structures of the aptamers were predicted using the online software RNAfold web server [38]. The simulations were done at default conditions with DNA parameters as energy parameters.

Results

Selecting for CSF biomarker-specific aptamers

Our initial synthesized pool of ssDNA sequences possessed a sequence diversity of 10^{16}. We started the SELEX protocol by capturing the ssDNA pool onto streptavidin beads, followed by extensively washing the beads with DPBS and serum to remove

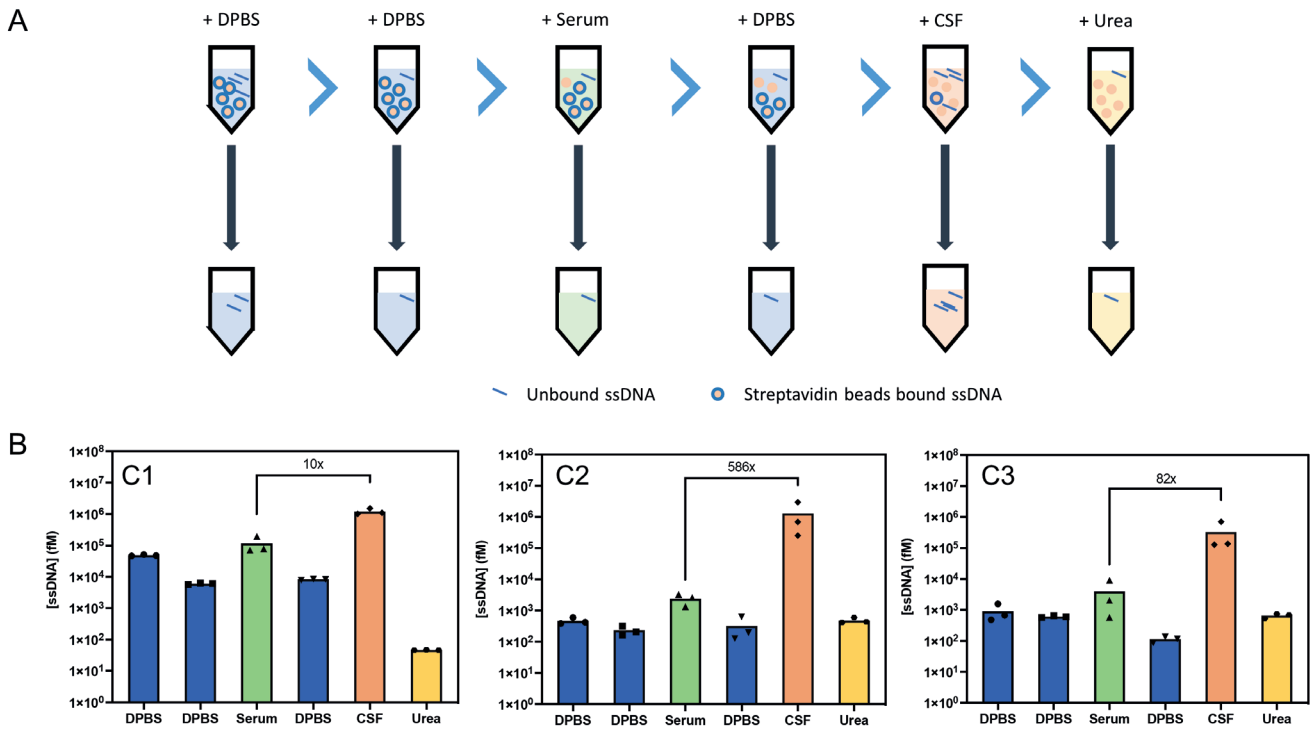


Figure 3. (A) qPCR experimental protocol. The ssDNA was bound to streptavidin beads and excess unbounded ssDNA was washed with DPBS twice. The remaining bounded ssDNA was washed with serum, followed by DPBS and then CSF. DNA samples were extracted from all washes and quantified by qPCR. (B) Quantified ssDNA concentrations for washes involving C1, C2, and C3. Concentrations of C1, C2, and C3 ssDNA were 10x, 586x, and 82x higher, respectively, in CSF as compared to serum washes.

sequences that were weakly bound or could respond to proteins (e.g., albumin) in human serum (Figure 1). We then introduced pure CSF obtained from lumbar drains of patients undergoing skull base surgery to allow interactions between CSF molecules and the remaining bead-bound ssDNA. We collected and amplified DNA sequences released in the presence of CSF, hypothesizing that they were enriched for ssDNAs that bind CSF-specific biomarkers and undergo a conformational change substantial enough to result in dissociation from the capture oligonucleotide.

Identifying enriched sequences

A total of 14 rounds of SELEX were performed to identify candidate CSF-specific aptamers. With the initial goal of identifying CSF biomarker-specific binders, we used either 5% BSA or 1% serum as the counterselection agents for the first 9 rounds of in vitro selection. For subsequent cycles (#10-14), we used high serum concentrations with the goal of delineating candidate aptamers that possess high specificity for CSF and discrimination against physiological contaminants present in serum. After 14 rounds of selection, we sequenced the library and bioinformatic analysis revealed several highly enriched sequences (Figure 2). In identifying candidate aptamers, two criteria were considered: (1) high sequence enrichment (i.e., reads per million [RPM]) and (2) greater sequence enrichment in CSF relative to serum. Among the top 10 sequence clusters, only the first 3 clusters (C1, C2, C3) met both criteria and were further characterized with qPCR.

Evaluating aptamer selectivity

To evaluate the binding capacity and selectivity of our candidate aptamers (C1-C3), we sequentially incubated the streptavidin bead-bound ssDNA in PBS, serum, and CSF per the protocol outlined in Figure 3. ssDNA that interacted with molecules in the solutions would unbind from the beads and fall into solution. Thus, by using qPCR on the resulting solution washes, we were able to quantify the amount of ssDNA that bound to the molecules in each solution. The PBS washes served as negative controls, and ssDNA that unbound due to these washes were likely due to passive dissocation from turbulent mixing and indicated poor binding strength. C1 and C2 demonstrated the highest ssDNA concentration in CSF. However, C1 exhibited relatively high ssDNA concentrations in serum, suggesting poor specificity for CSF. Therefore, when considering both binding affinity and specificity, qPCR results favored C2 and C3 as candidate aptamer sequences for further analysis.

Secondary structures for C2 and C3 sequences were predicted using RNAfold (Figure 4). Due to differences in their predicted

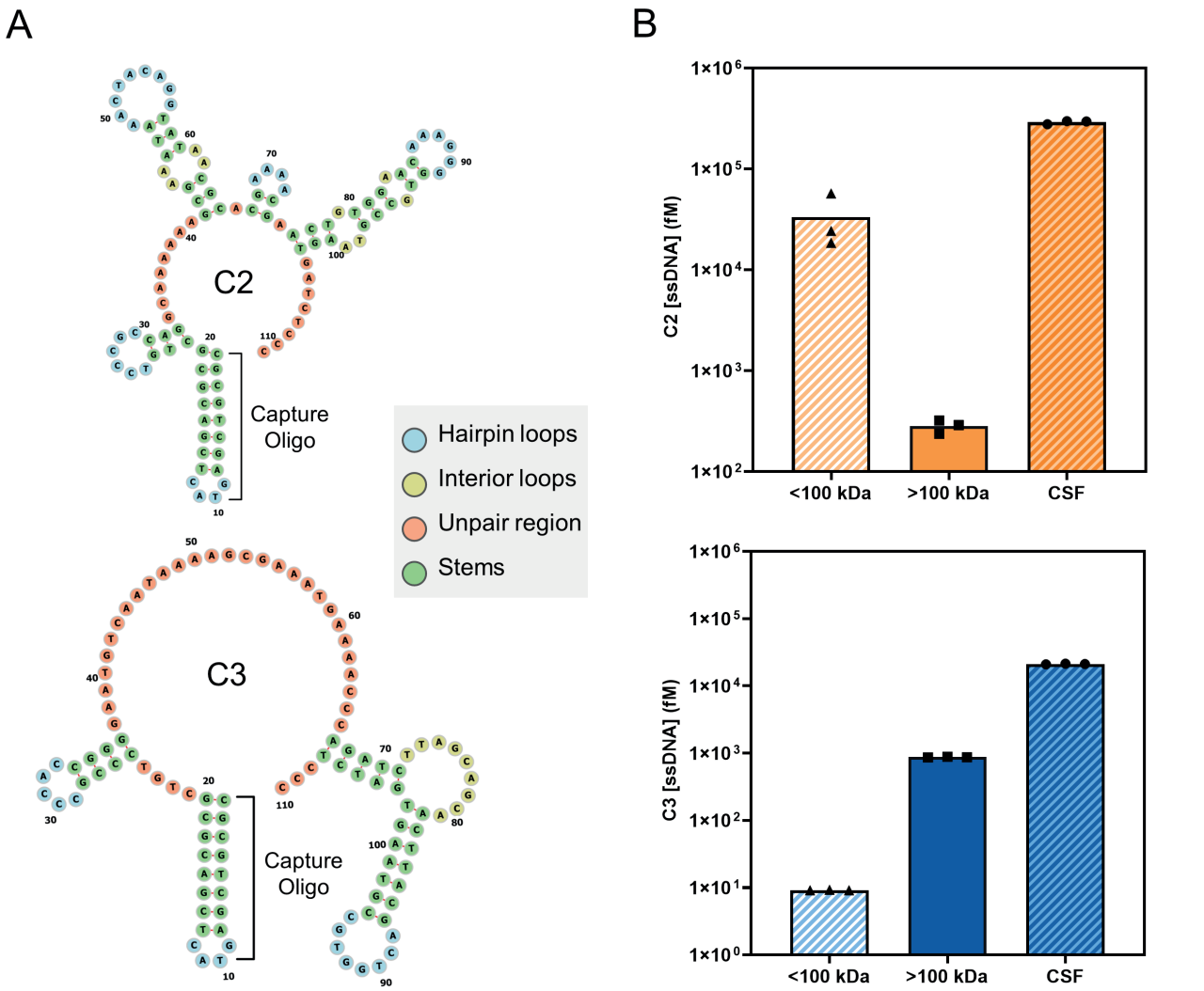


Figure 4. (A) Secondary structure predictions for C2 and C3 sequences demonstrating possible unpaired regions (orange) and sites of hairpin (blue) or interior loops (yellow). (B) Quantified ssDNA concentrations of C2 and C3 following washing with size-excluded (<100 kDa or >100kDa) fractionated or whole CSF. A higher concentration of C2 was detected in <100 kDa fractionated CSF than in >100 kDa fractionated CSF. In contrast, a higher concentration of C3 was detected in >100 kDa fractionated CSF than in <100 kDa fractionated CSF.

structures, we hypothesized that C2 and C3 may also bind to different target molecules. Using size exclusion filtration, whole CSF was fractionated into solutions consisting of molecules <100 and >100 kDa in size. Our candidate apatamers were then tested using samples of whole CSF, <100 kDa CSF, and >100 kDa CSF per the protocol in Figure 3. qPCR analysis suggested that C2 may favor molecules of <100 kDa while C3 may favor molecules of >100 kDa, implying separate targets (Figure 4).

Evaluating aptamer binding kinetics

To evaluate our aptamers' binding capacities and their propensity for conformational change in the presence of the target CSF biomarkers, we measured the FA in samples containing a constant aptamer concentration incubated with varying concentrations of CSF (Figure 5). A random 98-nt sequence containing the same 5'-terminus fluorophore and primer sequence was used as the negative control (NC). C2 and C3 demonstrated increasing anisotropy with higher concentrations of CSF, while the NC did not exhibit any significant anisotropy changes. Applying nonlinear regression on the data per the Langmuir Isotherm equation demonstrated apparent $K_{1/2}$ values of 5.0% and 14.1% CSF for C2 and C3, respectively. To validate our findings, we similarly measured FA in samples containing a constant aptamer concentration incubated with varying concentrations of 1% serum (Figure S1). A solution of 1% serum was used in order to simulate a similar protein concentration as CSF [39]. The negative control consisted of the same 5'-terminus fluorophore and primer sequence. Higher serum concentrations did not exhibit significant increases in FA for C2, C3, and the negative control.

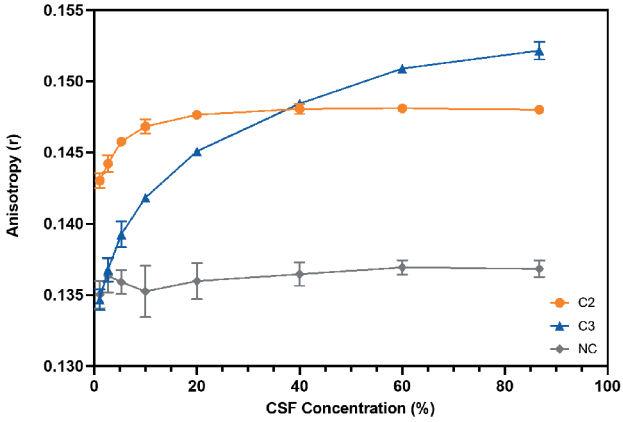


Figure 5. Fluorescence anisotropy of Cy3-labeled C2 and C3 ssDNA after 2-minute incubation with varying CSF concentrations. A random cy3-labeled 98-nt oligonucleotide uninvolved in the selection protocol was used as a negative control (NC). With higher CSF concentrations, C2 and C3 exhibited increases in anisotropy while the NC oligonucleotide did not.

Discussion

In this study, we utilized SELEX to discover conformation-switching DNA aptamers that could bind to CSF-specific biomarkers and discriminate against contaminating molecules from other physiological fluids (e.g., serum). After 14 cycles of in vitro selection, we identified two 98-nt ssDNA sequences that demonstrated favorable binding kinetics and selectivity for CSF compared to serum. Although recent efforts in CSF detection have implemented immunoassays, aptamers have been shown to possess significant advantages over antibodies due to their nucleic acid nature and target-induced structure-switching properties [40]. As they are chemically synthesized through an in vitro process, aptamers not only offer remarkable design flexibility, but are inexpensive to produce and are generated to a high degree of purity with little to no batch-to-batch variation [40–43]. Therefore, in discovering DNA aptamers that are highly selective for CSF-specific biomarkers, we have paved the way for developing cost-effective EAB biosensors capable of rapidly and accurately detecting CSF.

When developing an aptamer to be implemented for CSF detection, in addition to binding affinity, strength of binding and specificity for CSF biomarkers was critical. In our qPCR analysis, we found that the concentrations of C2 and C3 were significantly higher in the CSF wash compared to the DPBS and serum washes. The low aptamer concentrations detected after exposure to serum indicates that our aptamer did not effectively "switch off" and unbind from the streptavidin beads in the presence of serum-specific proteins (e.g., transferrin, albumin, thrombin). Importantly, the concentrations of ssDNA following serum washes were not significantly different from those after DPBS washes, suggesting that the low levels of detected ssDNA may have been due to passive dissociation of the aptamers from the streptavidin beads, potentially exacerbated by turbulent mixing during the washes. On the other hand, the higher aptamer concentrations following exposure to CSF indicates that the aptamers were highly selective for CSF-specific biomarkers. Additionally, low concentrations of ssDNA were found remaining on the streptavidin beads after CSF exposure, indicating that a low volume of CSF contained a sufficient concentration of target molecules to interact with and induce a conformational switch in most of the aptamers originally bound to streptavidin beads. The dose-dependent rise in FA indicated an increase in the concentration of aptamer-target complexes, thereby supporting that C2 and C3 undergo a conformational change when bound to CSF-specific biomarkers. This is an important characteristic for future implementation into an E-AB sensor, since a conformational switch and, hence, positional change in the MB moiety will be required to induce a detectable change in signal. Within the framework of an electrochemical sensor, MB's role as an electron donor becomes crucial. As this altered MB moiety comes into proximity with the gold substrate of the sensor to which the aptamer is affixed, it induces a notable change in the efficiency of electron transfer. This alteration is detectable as a change in electrical current, forming the basis for detecting the presence of CSF-specific biomarkers in an E-AB sensor's readout [44].

Interestingly, experiments using size-excluded CSF samples suggested that our aptamers may have a tendency to interact with different targets. Specifically, C2 appeared to favor molecules <100 kDa in size while C3 favored molecules larger than 100 kDa. These findings are in line with observations from FA analysis, which demonstrated a greater rise in anisotropy with aptamer-target complexes of C3 compared to C2. Since C2 and C3 are the same size, a greater change in anisotropy following target binding suggests a larger sized target. Although the identities of the aptamers' target molecules are not known, given that the estimated size of β2TF is ~78 kDa and BTP is 23-29 kDa, it is possible that C2 is interacting with one of these well-established CSF biomarkers [45,46]. Though, it is unclear if the target of C3, which exceeds 100 kDa, is an oligomer of β2TF or BTP or if it is a larger molecule that has yet to be established as a distinctive biomarker of CSF.

There are limitations in this study worth noting. CSF samples were taken directly from lumbar drains and were grossly clear, so the tested samples contained minimal contaminants (e.g., blood, mucus), as would be typically expected in a nasal drainage specimen. Therefore, the differences seen between negative controls and CSF samples are likely more pronounced than what would be observed in clinical application. However, given our aptamers' low apparent $K_{1/2}$ and strong selectivity for CSF relative to serum, future analyses on CSF-containing rhinorrhea samples are still likely to exhibit 1 to 2 orders of magnitude higher signal than negative controls. The presence of mucus in

nasal drainage samples may also pose a challenge, since nasal mucus contains enzymes that may degrade DNA and CSF proteins. Future studies would benefit from examining the effects of mucus on aptamer and CSF sample stability.

Conclusion

In this study, we conducted 14 cycles of in vitro selection using human CSF samples to uncover two conformation-switching 98-nt ssDNA aptamers capable of selectively binding to CSF-specific biomarkers. qPCR and FA analysis demonstrated favorable binding kinetics for CSF detection and strong discrimination against similar molecules found in human serum. Integration of these CSF-specific aptamers into an E-AB sensor may serve as an accurate and cost-effective diagnostic modality for rapidly detecting CSF leaks.

Acknowledgement

This work was preliminarily presented at the 42nd Congress of the International Society of Inflammation and Allergy of the Nose and 24th Congress of the International Rhinologic Society on April 4, 2024 in Tokyo, Japan.

Authors' contributions

Data acquisition: AA, XC; Data analysis: AA, XC, BL; Drafting article: AA, XC, BL, FPKH, AL, MK, ECK; Final approval: AA, XC, BL, FPKH, AL, MK, ECK.

Funding

This work was supported in part by the UCI Institute for Clinical and Translational Science Pilot Studies Award, International Society of Inflammation and Allergy of the Nose New Investigator Research Grant, North American Skull Base Society Research Grant, UCI Interim COVID-19 Research Recovery Program Award, UCI School of Medicine New Investigator Faculty Research Grant, and the American Rhinologic Society New Investigator Research Grant.

Conflicts of interest

The authors declare that there is no relevant conflict of interest.

Ethics approval

This study was conducted in accordance with the Institutional Review Boards at University of California, Irvine, Orange, CA, USA.

Availability of data and materials

Data used in this study is available from the corresponding author (ECK) on reasonable request.

References

1. Friedman JA, Ebersold MJ, Quast LM. Post-traumatic cerebrospinal fluid leakage. World J Surg. 2001;25(8):1062-1066.
2. Loew F, Pertuiset B, Chaumier EE, Jaksche H. Traumatic, spontaneous and postoperative CSF rhinorrhea. Adv Tech Stand Neurosurg. 1984;11:169-207.
3. Prosser JD, Vender JR, Solares CA. Traumatic cerebrospinal fluid leaks. Otolaryngol Clin North Am. 2011;44(4):857-873.
4. Daudia A, Biswas D, Jones NS. Risk of meningitis with cerebrospinal fluid rhinorrhea. Ann Otol Rhinol Laryngol. 2007;116(12):902-905.
5. Kono Y, Prevedello DM, Snyderman CH, et al. One thousand endoscopic skull base surgical procedures demystifying the infection potential: incidence and description of postoperative meningitis and brain abscesses. Infect Control Hosp Epidemiol. 2011;32(1):77-83.
6. Proulx N, Fréchette D, Toye B, Chan J, Kravcik S. Delays in the administration of antibiotics are associated with mortality from adult acute bacterial meningitis. QJM Mon J Assoc Physicians. 2005;98(4):291-298.
7. Ricketti AJ, Cleri DJ, Porwancher RB, Panesar M, Villota FJ, Seelagy MM. Cerebrospinal fluid leak mimicking allergic rhinitis. Allergy Asthma Proc. 2005;26(2):125-128.
8. Schievink WI. Spontaneous spinal cerebrospinal fluid leaks and intracranial hypotension. JAMA. 2006;295(19):2286-2296.
9. Kovalerchik O, Mady LJ, Svider PF, et al. Physician accountability in iatrogenic cerebrospinal fluid leak litigation. Int Forum Allergy Rhinol. 2013;3(9):722-725.
10. Borg A, Kirkman MA, Choi D. Endoscopic endonasal anterior skull base surgery: a systematic review of complications during the past 65 Years. World Neurosurg. 2016;95:383-391.
11. Reddy M, Baugnon K. Imaging of cerebrospinal fluid rhinorrhea and otorrhea. Radiol Clin North Am. 2017;55(1):167-187.
12. Pang J, Meller L, Nguyen C, et al. Conventional clinical signs and symptoms are poor predictors of postoperative cerebrospinal fluid leak: a single-center cohort perspective. Clin Otolaryngol. 49(5):621-632.
13. Oakley GM, Alt JA, Schlosser RJ, Harvey RJ, Orlandi RR. Diagnosis of cerebrospinal fluid rhinorrhea: an evidence-based review with recommendations. Int Forum Allergy Rhinol. 2016;6(1):8-16.
14. Görögh T, Rudolph P, Meyer JE, Werner JA, Lippert BM, Maune S. Separation of β2-transferrin by denaturing gel electrophoresis to detect cerebrospinal fluid in ear and nasal fluids. Clin Chem. 2005;51(9):1704-1710.
15. Warnecke A, Averbeck T, Wurster U, Harmening M, Lenarz T, Stöver T. Diagnostic relevance of beta2-transferrin for the detection of cerebrospinal fluid fistulas. Arch Otolaryngol--Head Amp Neck Surg. 2004;130(10):1178-1184.
16. McCudden CR, Senior BA, Hainsworth S, et al. Evaluation of high resolution gel β(2)-transferrin for detection of cerebrospinal fluid leak. Clin Chem Lab Med. 2013;51(2):311-315.
17. Brown KJ, Vanderver A, Hoffman EP, Schiffmann R, Hathout Y. Characterization of transferrin glycopeptide structures in human cerebrospinal Fluid. Int J Mass Spectrom. 2012;312:97-106.
18. Nandapalan V, Watson ID, Swift AC. Beta-2-transferrin and cerebrospinal fluid rhinorrhoea. Clin Otolaryngol Allied Sci. 1996;21(3):259-264.
19. Abuabara A. Cerebrospinal fluid rhinorrhoea: diagnosis and management. Med Oral Patol Oral Cirugia Bucal. 2007;12(5):E397-400.
20. Mantur M, Łukaszewicz-Zając M, Mroczko B, et al. Cerebrospinal fluid leakage--reliable diagnostic methods. Clin Chim Acta Int J Clin Chem. 2011;412(11-12):837-840.
21. Korem M, Ovadia H, Paldor I, et al. False negative β-2 transferrin in the diagnosis of cerebrospinal fluid leak in the presence of Streptococcus pneumoniae. Laryngoscope. 2015;125(3):556-560.
22. Eide JG, Mason W, Ray A, Carey J, Cook B, Craig JR. Systematic review of errors on beta-2 transferrin gel electrophoresis testing of rhinorrhea and otorrhea. Int Forum

Allergy Rhinol. 2024; 14(6):1016-1025.
23. Carey PH, Lobo BC, Gebhard MP, et al. Review-opportunities for rapid, sensitive detection of troponin and cerebral spinal fluid using semiconductor sensors. J Electrochem Soc. 2019;167(3):37507.
24. Papadea C, Schlosser RJ. Rapid method for beta2-transferrin in cerebrospinal fluid leakage using an automated immunofixation electrophoresis system. Clin Chem. 2005;51(2):464-470.
25. Kita AE, Bradbury DW, Taylor ZD, Kamei DT, St. John MA. Point-of-care cerebrospinal fluid detection. Otolaryngol Neck Surg. 2018;159(5):824-829.
26. Bradbury DW, Kita AE, Hirota K, St John MA, Kamei DT. Rapid diagnostic test kit for point-of-care cerebrospinal fluid leak detection. SLAS Technol. 2020;25(1):67-74.
27. Dietzel J, Krebs A, Böttcher D, et al. Beta-trace protein in ascites and pleural effusions: limits of CSF leakage detection. J Neurotrauma. 2012;29(9):1817-1820.
28. Michaelides EM, Kuang H, Pieribone VA. Dickkopf-related protein 3 as a sensitive and specific marker for cerebrospinal fluid leaks. Otol Neurotol. 2016;37(3):299-303.
29. Carey PH, Yang J, Ren F, et al. Fast cerebrospinal fluid detection using inexpensive modular packaging with disposable testing strips. J Electrochem Soc. 2019;166(8):B708-B712.
30. Xian M, Chiu CW, Carey PH, et al. Digital biosensor for human cerebrospinal fluid detection with single-use sensing strips. J Vac Sci Technol B. 2022;40(2):23202.
31. Rhie A, Kirby L, Sayer N, et al. Characterization of 2'-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion. J Biol Chem. 2003;278(41):39697-39705.
32. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. Nature. 1990;346(6287):818-822.
33. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science. 1990;249(4968):505-510.
34. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics. 2014;30(5):614-620.
35. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-359.
36. Alam KK, Chang JL, Burke DH. FASTAptamer: A bioinformatic toolkit for high-throughput sequence analysis of combinatorial selections. Mol Ther Nucleic Acids. 2015;4(3):e230.
37. Bloomfield IG, Johnston IH, Bilston LE. Effects of proteins, blood cells and glucose on the viscosity of cerebrospinal fluid. Pediatr Neurosurg. 1998;28(5):246-251.
38. Lorenz R, Bernhart SH, Höner zu Siederdissen C, et al. ViennaRNA Package 2.0. Algorithms Mol Biol AMB. 2011;6:26.
39. Schilde LM, Kösters S, Steinbach S, et al. Protein variability in cerebrospinal fluid and its possible implications for neurological protein biomarker research. PLoS ONE. 2018;13(11):e0206478.
40. Chen A, Yang S. Replacing antibodies with aptamers in lateral flow immunoassay. Biosens Bioelectron. 2015;71:230-242.
41. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin Chem. 1999;45(9):1628-1650.
42. Toh SY, Citartan M, Gopinath SCB, Tang TH. Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. Biosens Bioelectron. 2015;64:392-403.
43. Song S, Wang L, Li J, Fan C, Zhao J. Aptamer-based biosensors. TrAC Trends Anal Chem. 2008;27(2):108-117.
44. Lai RY, Plaxco KW, Heeger AJ. Aptamer-based electrochemical detection of picomolar platelet-derived growth factor directly in blood serum. Anal Chem. 2007;79(1):229-233.
45. Schwab S, Kleine CE, Bös D, et al. Beta-trace protein as a potential biomarker of residual renal function in patients undergoing peritoneal dialysis. BMC Nephrol. 2021;22:87.
46. Luo RY, Pfaffroth C, Yang S, et al. Study of β1-transferrin and β2-transferrin using microprobe-capture in-emitter elution and high-resolution mass spectrometry. Sci Rep. 2023;13:14974.

Edward C. Kuan, MD, MBA
University of California, Irvine
101 The City Drive South
Orange, CA 92868
USA

E-mail: eckuan@uci.edu

Arash Abiri[1,2,*], Xinlei Chen[1,*], Brandon Latifi[3], Frank P.K. Hsu[4], Andrej Lupták[3], Michelle Khine[1], Edward C. Kuan[2,4,#]

[1] Department of Biomedical Engineering, University of California Irvine, Irvine, CA, USA
[2] Department of Otolaryngology-Head and Neck Surgery, University of California, Irvine (UCI), CA, USA
[3] Department of Pharmaceutical Sciences, University of California, Irvine, CA, USA
[4] Department of Neurological Surgery, University of California, Irvine, CA, USA

Rhinology 63: 1, 103 - 112, 2025
https://doi.org/10.4193/Rhin24.341

Received for publication:
July 28, 2024
Accepted: September 29, 2024

Assocociate Editor:
Ahmad Sedaghat

* contributed equally to this work

This manuscript contains online supplementary material

SUPPLEMENTARY MATERIAL

Table S1. DNA Sequences

DNA Library:	5′-ACTCGACGCGCTGTCCC-N63-GGTGCCGTAAGTGATCTCCC-3′
Capture probe:	/5Biosg//iSp18//iSp18//iSp18/CGCGTCGAGT-3′
MB primer:	/5MeBIN/ACTCGACGCGCTGTCCC-3′
Cy3 primer:	/5Cy3/ACTCGACGCGCTGT CCC-3′
Forward primer:	5′-GGGAGATCACTTACGGCACC-3′
Reverse primer:	5′-CGACGCGCTGTCCCG-3′

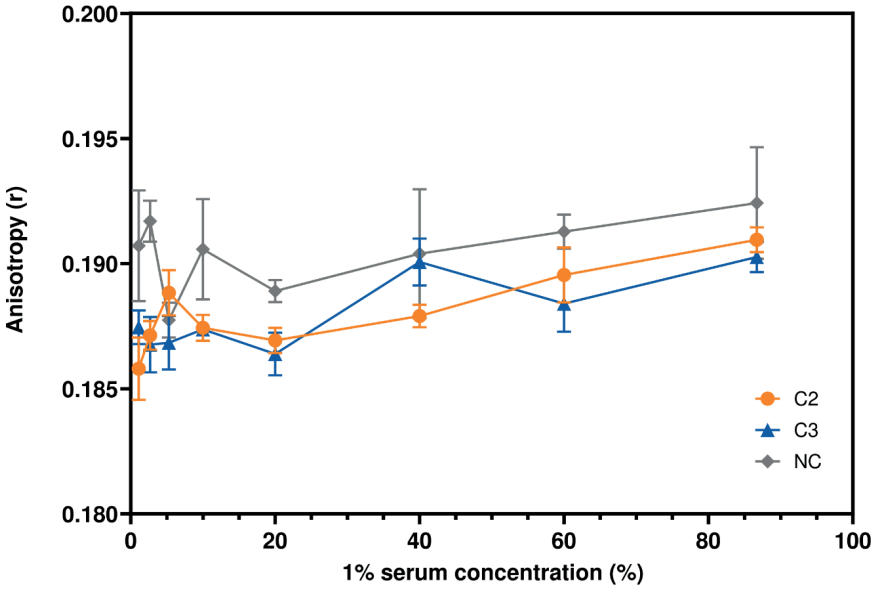


Figure S1. Fluorescence anisotropy of Cy3-labeled C2 and C3 ssDNA after 2-minute incubation with varying 1% serum concentrations. A random cy3-labeled oligonucleotide uninvolved in the selection protocol was used as a negative control (NC). C2, C3, and NC did not exhibit significant increases in anisotropy with higher serum concentrations.