Journal of The Electrochemical Society, **166** (8) B708-B712 (2019)
0013-4651/2019/166(8)/B708/5/$38.00 © The Electrochemical Society

Fast Cerebrospinal Fluid Detection Using Inexpensive Modular Packaging with Disposable Testing Strips

Patrick H. Carey IV,[1,*] Jiancheng Yang,[1,*] Fan Ren,[1,,z] Yu-Te Liao,[2] Chin-Wei Chang,[3] Jenshan Lin,[3] Stephen J. Pearton,[4,**] Brian Lobo,[5] and Marino E. Leon[6]**

[1]*Department of Chemical Engineering, University of Florida, Gainesville, Florida 32611, USA*
[2]*Department of Electrical and Computer Engineering, National Chiao Tung University, Hsinchu, Taiwan*
[3]*Deparment of Electrical and Computer Engineering, University of Florida, Gainesville, Florida 32611, USA*
[4]*Department of Materials Science and Engineering, University of Florida, Gainesville, Florida 32611, USA*
[5]*Department of Otolaryngology, College of Medicine, University of Florida, Gainesville, Florida 32611, USA*
[6]*Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida 32611, USA*

This report details the use of a Si Metal Oxide Semiconductor Field Effect Transistor (MOSFET) with the gate connected to a disposable antibody-functionalized electrode for the detection of β-2-transferrin (β2T) as a marker of cerebral spinal fluid (CSF). Through the design of a Si based PCB the need for GaN High Electron Mobility Transistors has been mitigated and brings the cost of production for commercial application closer to realization. Through the use of a double pulse method to the test electrode and the drain of the Si MOSFET, ionicity of the test solution is no longer a concern as a double layer is produced and perturbed on the test electrode giving rise to current changes related to the concentration of the biomarker. Additionally, the PCB provides connections for an oscilloscope readout or an onboard display entirely removing the need for any expensive semiconductor parameter analyzers. Clinically, the cutoff for β2T as indicator of CSF leakage has traditionally been limited to ∼2 μg/mL and takes several hours; with this newly designed board, CSF was detected at levels from 0.1 ng/mL to 100 μg/mL within 5 minutes, greatly improving the detection limits and feedback time for medical professionals.
 [DOI: 10.1149/2.1131908jes]

Manuscript submitted January 22, 2019; revised manuscript received April 22, 2019. Published May 16, 2019.

Cerebrospinal fluid (CSF) is a liquid found through the brain's ventricles and around the brain and spinal cord. CSF acts as a liquid transport for chemicals to and from the brain and maintains the cranial pressure, which acts as a cushion to the brain in case of sudden shocks. As CSF can act as a waste vehicle for neurons and other nervous system cells it must be replenished continuously. In a normal human adult there is 125 to 150 mL of CSF at one time, which is replenished every 6 hours, so approximately 600–700 mL of CSF is produced daily and thus leaks can be of significant quantity.[1–3] A CSF leak is a serious complication that can result from trauma to the skull base, otolaryngology (ENT) surgery, lumbar puncture, and on rare occasion purely spontaneously. The primary concern as a result of a leak is intracranial infection which left untreated can be deadly. Common symptoms include headache, nasal drainage, ear drainage, fever, and tinnitus. CSF leaks are traditionally detected through collection of the nasal/ear fluid, which undergoes analysis for the protein β-2-transferrin (β2T).[4–10] The β-2 conformation of transferrin is carbohydrate-free and exclusively found in CSF, not in blood, mucus or tears. It can also be found in patients with perilymph fluid leaks.[11] Once a CSF leak has been detected establishing the location of the leak is the next step to determine whether endonasal endoscopic surgery is recommended. High-Resolution Computed Tomographic (HRCT) scans are most used for identification of the leak location as it provides 1 to 2 mm sections in the coronal and axial planes with algorithms for identification of bone to best identify the skull base defects which are causing the leaks.[12–14] Surgery is used to temporarily seal the leak so that the bone can repair itself and prevent infection until full recovery has occurred.[15–17]

There are two primary methods of detection for a CSF leak, immunofixation electrophoresis (IFE) and enzyme-linked immunosorbent assay (ELISA).[18–21] IFE relies upon the separations of proteins by their molecular weight and as the β-2 conformation will traverse farther along an acidic gel than the β-1 due to being desialated. Papadea et al. demonstrated consistent results with IFE down to 2 μg/mL using samples from patient, but this result required a 2.5 hour testing period not expedient enough for real time feedback during ENT surgeries.[20] Additionally, to achieve good sensitivity and handle the inherently low concentration of β-2 transferrin in CSF, laboratory procedures have required samples to be concentrated by as much as 10-fold or the sample to be run in duplicate to ensure accurate detection.

To alleviate the slow turn-around times of hospital laboratories and fairly limited lower limits of detection, there has been strong interest in electronic detection methods for proteins, viruses, or small molecules using biologically functionalized field effect transistors (FETs). There has been much previous work exploring bio-functionalized AlGaN/GaN high electron mobility transistors (HEMTs) due to the excellent sensing characteristics from the high-density electron channel located near -surface (∼25 nm).[22–24] For sensing of proteins one of the major concerns is the high ionicity of the test solution (blood/serum). The high ionicity can cause charge screening effects where the Debye screening length is shorter than that of the antibody/protein. To circumvent this issue Yang et al, Hsu et al., and Sarangadharan et al. utilized a double pulse measurement which provokes a spring-like response of the antibody-protein complex that can be then sensed regardless of the highly ionic solutions.[25–28] Yang et al. pushed to the next step of modularization by externalizing the sensing component from the HEMT, making the device reusable as a testing strip replaced each time. However, a main limitation of all these works is the use of AlGaN/GaN HEMTs due to the high cost of the substrates as they are prepared through low throughput methods, such as metal organic chemical vapor deposition, rather than bulk growth methods like the Czochralski method for silicon.

In this work a disposable testing slide was externally integrated with a PCB board with a Si MOSFET to detect CSF from 0.1 ng/mL to 100 μg/mL. The PCB with off-self components for pulse generation, voltage adjustment and a Si MOSFET was designed to simplify the testing setup and drive the cost down by removing the need for expensive semiconductor parameter analyzers.

Experimental

A disposable sensing unit was fabricated on a disposable glass slides with the Ni/Au based (20/80 nm) metal electrodes using E-beam evaporation and a standard lift-off process. To passivate the electrodes, SU-8 was employed and opened on the sensing ends of the electrodes and on the contact windows. To immobilize anti β-2-transferrin antibody on the metal electrodes, the electrodes were treated with thioglycolic acid (TGA) for 12 hours at room temperature. Excess TGA was rinsed off using deionized (DI) water. The Au-S bond will form a strong bond with no additional reagents

*Electrochemical Society Student Member.
**Electrochemical Society Fellow.
[z]E-mail: fren@ufl.edu

needed.[24] The device was then incubated in a PBS solution of CSF monoclonal antibody. Anti β-2-transferrin antibody was reacted at 4°C for 4 hours on the sensing ends of the electrodes, not the contact pads. The sensing unit was then rinsed with DI water and phosphate buffer saline (PBS) to remove any excess unbound antibody. The sensor was stored at 4°C in PBS when not in use. The binding efficiency of the antibody to the gold surface is entirely dependent on a case-by-case basis. If poor binding efficiency is noted, i.e. poor sensitivity to the target protein, a two-step chemical reaction can be used to improve and activate the surface carboxylic acids.[23] This can be achieved by submerging the device first in a 0.1 mM solution of N,N'-dicyclohexylcarbodi-imide in acetonitrile for 30 minutes and then in a 0.1 mM solution of N-hydroxysuccinimide in dry acetonitrile for 1 hour. These functionalization steps resulted in the formation of succinimidyl ester groups on gold of the sensing electrodes. Then incubating the device with the mAB of choice. For this study, these two steps were not needed as good sensitivity was noted. Previously, we have established stability of the antibody on the sensor surface for up to 9 months after initial fabrication without using these two steps[29] To prevent non-specific surface binding interactions between unbound TGA and any competing off-target proteins, the TGA was terminated by treatment with ethanolamine after incubation with the mAB.

Human pooled CSF was serially diluted from 100 μg/mL to 0.1 ng/mL in pH 7.4 PBS and 1 wt% bovine serum albumin (BSA). Bovine serum albumin serves two purposes: to stabilize the proteins in solutions and to act as control for non-specific binding effects as the BSA is many orders of magnitude more concentrated than the target protein in solution. We recognize that the concentration of CSF is not the concentration of β2T, which is our target protein, β2T concentration is actually much lower as it is only a minor constituent of CSF. Total protein content of CSF has been reported from 150 to 500 mg/L in healthy adults. To test the CSF sensor, a CSF solution was applied to the sensing electrode and allowed to bind to the sensor surface for 5 minutes prior to measurement.

Results and Discussion

PCB design and setup.—Figure 1 shows the generalized PCB design and an optical image of the PCB connected to the sensing

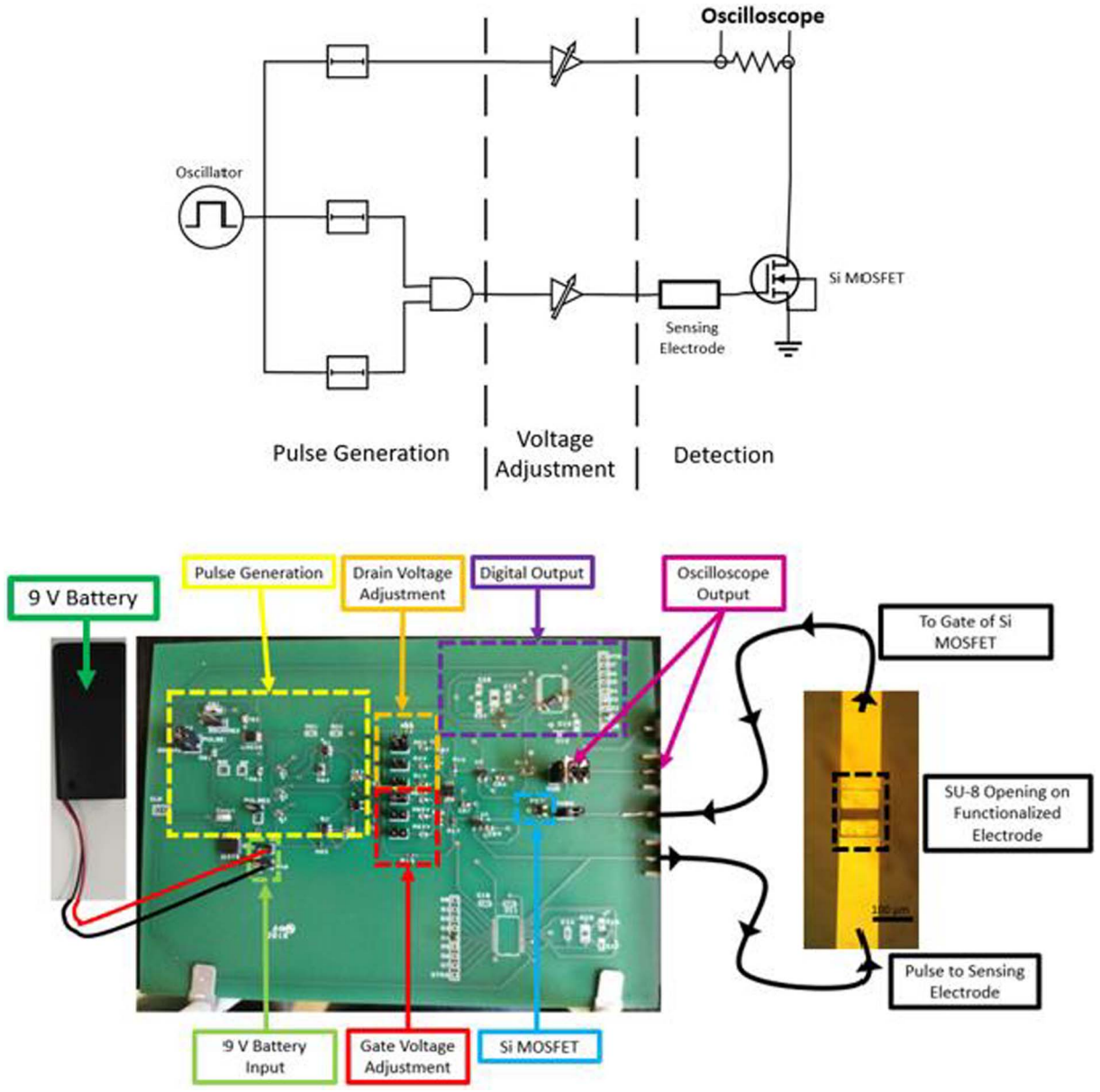

Figure 1. PCB general overview schematic and optical image.

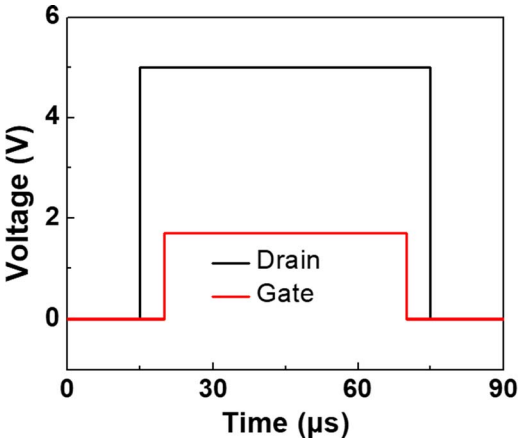

Figure 2. Synchronous pulse of the Si D-FET drain and gate.

electrode. The circuit includes three main functional blocks: pulse generation, voltage level adjustment, and detection. There are two synchronous pulses applied to the D-mode Si MOSFET. The first is a 60 µs pulse to the drain, turning the device on, and 5 µs later a synchronous pulse is applied to the gate for 50 µs, Figure 2 illustrates the synchronous pulses.

In the pulse generation block, a mono-stable oscillator followed by delay lines and gate generate pulses that are overlapping but with different widths. The voltage level adjustment allows for on PCB varying the applied gate voltage to the sensing chip in order to optimize sensing on a protein-by-protein basis, rather than designing a new PCB for every sensing target. Furthermore, depending on a protein's length, net charge, and folding, the applied voltage needs to be roughly tuned such that the protein-antibody complex is perturbed and undergoes a spring-like relaxation. If insufficient voltage is supplied there will be no perturbation of the protein-antibody complex. If excessive voltage is applied there may be full elongation and dissociation of the protein-antibody complex. In either case sensing of the target protein will not occur repeatably. The drain current can also be adjusted in order to tune the device for maximum transconductance. For this study, $V_d = 5$ V and $V_g = 1.7$ V were used. Detection of the target protein is monitored by an oscilloscope via the drain current through a 50 Ω resistor placed before the drain of the D-FET. Additionally, in order to ensure detection at the lower limits of ng/mL, traces in the circuit were kept short in order to reduce noise from coupling.

Sensing.—To screen out defective sensors, PBS reference runs were performed three times subsequently. All three measurements were required to match for the sensor to be considered usable, Figure 3a contains an example output. Time dependent detection of CSF dilutions from 0.1 ng/mL to 100 ng/mL are shown in Figure 3b. The bend of the curves, Fig. 3b, is the dynamic drain current response due to the double spring-like response of the perturbed antibody-protein complex.[26] Of note to achieve such low sensitivity to the sub ng/mL range, noise of the designed PCB must be considered. From testing, we found that it was necessary to use a low noise DC power source, in our case a 9V battery, rather than a DC power source which was plugged into a standard 60 Hz wall outlet. This issue will be mitigated in future variations of our PCB by utilizing appropriate filters. Additionally, the presented response is the average of 16 measurements, averaging of more measurements would lead to a smoother curve with less noise.

Average gains across five separate sensors are shown in Figure 4, to demonstrate the reproducibility of such tests. Again it is important to remember, the concentration is of CSF as a whole, while we are truly only targeting the β2T which is a minor component of the proteins of CSF. Normal CSF contains < 1 g of all types of present proteins per 1 L of CSF; thus the actual tested concentration for β2T is at least 1000X fold more diluted than the stated CSF. Additionally, the

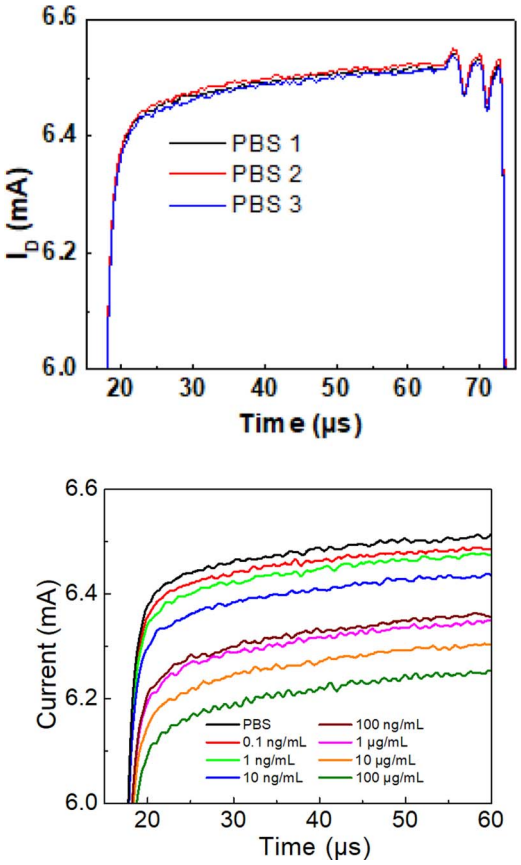

Figure 3. (a). Stability of sensor in PBS across three separate applications of PBS indicates low noise and eligibility for use in testing of CSF solutions and (b) time-dependent device current for varying CSF concentration from 0.1 ng/mL to 100 µg/mL.

sensor has not reached saturation even at a very high concentration of 100 µg/mL of CSF. The antibody sensor coverage was calculated to be approximately 5×10^6 antibody units on the sensing area.

Modelling of sensor gain.—The antigen and antibody binding occurs through an active process which is reversible. Previous modelling attempts by Yang et al. have used small molecule approximations, Langmuir Extended Isotherm, which provided good fits to relate the

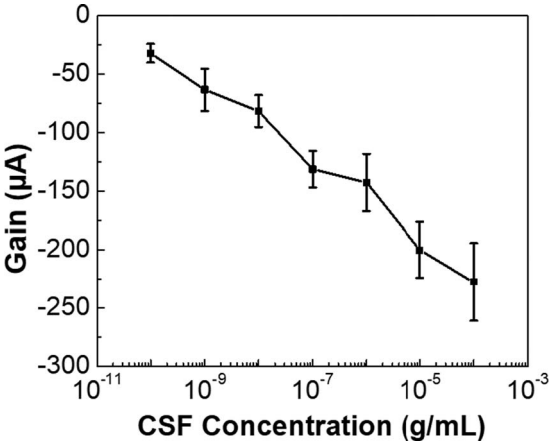

Figure 4. Average gain of CSF sensors from 0.1 ng/mL to 100 µg/mL.

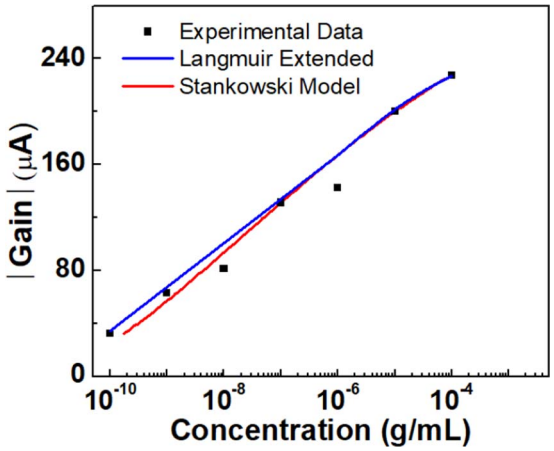

Figure 5. Langmuir-Extended and Stankowski model of absolute sensor gain as a function of test solution concentration.

gain of the sensor to target concentration.[25,26,30]

$$\Delta I = \frac{q_m b[C]^{\eta}}{1 + b[C]^{\eta}}$$

where q_m and b are Langmuir constants, [C] is the concentration of the target in the test solution, and η is related to the spread of the energy distribution of the target. However, the small molecule approximation is not fully accurate when discussing antibodies and proteins as their sizes in the 100's of kDa, thus steric hindrance is a serious consideration, especially when packed tightly on a surface. Additionally, with such large charged structures there will be electrostatic attraction and repulsion between neighboring ligands and between the functionalized surface of the sensor to the target protein in solution. For this purpose, a relation between solution concentration of the target protein and the fraction of the sensor surface which is bound was proposed by Stankowski et al.:

$$[C] = \frac{1}{K_0} \cdot \frac{\theta}{1-\theta} \cdot \exp\left(\frac{\theta}{1-\theta} + 2a\theta\right)$$

where K_0 is the effective initial rate constant of the antibody-protein binding, θ is the surface coverage, and a is a measure of the electrostatic repulsion of bound proteins on the sensor.[31,32] The exponential term contains the two competing effects of steric hindrance and electrostatic repulsion. As coverage approaches saturation ($\theta \rightarrow 1$), the steric term ($\frac{\theta}{1-\theta}$) will become dominant, asymptotic, as the protein covered surface is tightly packed and reduces the likelihood of free proteins successfully entering and binding to free antibodies on the sensor.

Experimental data was fit to both models and showed good agreement between the two, Figure 5. Agreement of the two models can be expected under low to medium coverage conditions. The Langmuir-Extended model can be expected to provide a good fit in regimes of low coverage of the sensor surface with the antibody where steric hindrance between neighboring proteins has not yet becoming a limiting factor. From the Langmuir model, the following constants were extracted $q_m = 311.8\ \mu A$, $b = 19.69\ (L/g)^{0.215}$, and $\eta = 0.215$. Of note q_m is the maximum expected current change in our sensor at saturation. The Stankowski model provides some further elucidation on the sensor functionality and underlying mechanisms. The exact coverage of the sensor is not directly known but can be approximated by the change in sensor current with concentration such that:

$$\theta = \frac{n}{i} \sim \frac{\Delta I}{\Delta I_{max}}$$

where n is the number of bound proteins to the sensor surface, and i is the total number of available antibodies on the sensor surface, ΔI is the experimental value of change in current, and ΔI_{max} is the change in current when the sensor surface has become saturated with the target protein. From the modeled fit $K_0 \sim 2.57 \times 10^9$ mL/g, $\Delta I_{max} = 319\ \mu A$, and $a = 6.33$. The positive value of a indicates a mild repulsion between antibody bound proteins. This repulsion is dominant at low to medium coverages of the sensor, but as the proteins begin to tightly pack on the surface steric hindrance will become dominant. Interestingly, the predicted maximum current change of $q_m = 311.8\ \mu A$ and $\Delta I_{max} = 319\ \mu A$ from the two models are similar and this provides some further confirmation of each result.

Conclusions

In conclusion, β2T as a marker of cerebral spinal fluid (CSF) which is released when damages to the skull or spinal column have been incurred has been successfully detected from concentrations of 0.1 ng/mL to 100 μg/mL. This CSF testing method provides for selective sensing of β2T through use of traditional monoclonal antibodies. Both the Langmuir Extended Isotherm and Stankowski model were employed to fit the experimental data and describe sensor performance. Design and implementation of an external PCB has been successfully implemented with disposable testing strips in the process of bringing a true point of care detection platform to all hospital personnel. As this test is able to detect β2T at very low concentrations; the specificity of this test should be a further study with human test subjects.

ORCID

Patrick H. Carey IV https://orcid.org/0000-0002-8826-3977
Fan Ren https://orcid.org/0000-0001-9234-019X
Jenshan Lin https://orcid.org/0000-0002-7439-9259
Stephen J. Pearton https://orcid.org/0000-0001-6498-1256

References

1. W. Pardridge, W. Oldendorf, P. Cancilla, and H. Frank, "Blood-Brain Barrier: Interface Between Internal Medicine and the Brain", *Annals of Internal Medicine*, **105**(1), 82 (1986).
2. P. Ballabh, A. Braun, and M. Nedergaard, "The blood–brain barrier: an overview", *Neurobiology of Disease*, **16**(1), 1 (2004).
3. J. Iliff, S. Goldman, and M. Nedergaard, "Implications of the discovery of brain lymphatic pathways", *The Lancet Neurology*, **14**(10), 977 (2015).
4. G. Oberascher, "Cerebrospinal fluid otorrhea – new trends in diagnosis", *Otology & Neurotology*, **9**(2), 102 (1988).
5. A. Warnecke, T. Averbeck, U. Wurster, M. Harmening, T. Lenarz, and T. Stöver, "Diagnostic Relevance of β2-Transferrin for the Detection of Cerebrospinal Fluid Fistulas", *Archives of Otolaryngology–Head & Neck Surgery*, **130**(10), 1178 (2004).
6. G. Bachmann and O. Michel, "Clinical Experience with Beta-Trace Protein as a Marker for Cerebrospinal Fluid", *Annals of Otology, Rhinology & Laryngology*, **109**(12), 1099 (2000).
7. G. Vihapure, K. Agarwal, and V. Dokania, "Retrospective Study of Endoscopic Management of CSF Rhinorrhoea- A Case Series", *Journal of Evolution of Medical and Dental Sciences*, **6**(30), 2473 (2017).
8. C. Meco and G. Oberascher, "Comprehensive Algorithm for Skull Base Dural Lesion and Cerebrospinal Fluid Fistula Diagnosis", *The Laryngoscope*, **114**(6), 991 (2004).
9. J. Zapalac, B. Marple, and N. Schwade, "Skull Base Cerebrospinal Fluid Fistulas: A Comprehensive Diagnostic Algorithm", *Otolaryngology-Head and Neck Surgery*, **126**(6), 669 (2002).
10. C. Schnabel, E. Di Martino, J. Gilsbach, D. Riediger, A. Gressner, and D. Kunz, "Comparison of β-2-Transferrin and β -Trace Protein for Detection of Cerebrospinal Fluid in Nasal and Ear Fluids", *Clinical Chemistry*, **50**(3), 661 (2004).
11. O. Delaroche, P. Bordureb, E. Lippert, and M. Sagnieza, "Perilymph detection by β2-transferrin immunoblotting assay. Application to the diagnosis of perilymphatic fistulae", *Clinica Chimica Acta*, **245**(1), 93 (1996).
12. B. Mostafa and A. Khafagi, "Combined HRCT and MRI in the Detection of CSF Rhinorrhea", *Skull Base*, **14**(3), 157 (2004).
13. A. Agbetoba, B. Delman, J. Bederson, and S. Govindaraj, "Use of HRCT-MRI Fusion Imaging in the Detection of Cerebrospinal Fluid Leaks", *Otolaryngology-Head and Neck Surgery*, **147**(2), P257 (2012).
14. N. Vemuri, L. Karanam, V. Manchikanti, S. Dandamudi, S. Puvvada, and V. Vemuri, "Imaging review of cerebrospinal fluid leaks", *Indian Journal of Radiology and Imaging*, **27**(4), 441 (2017).
15. J. Park, J. Choi, Y. Kim, S. Kim, and Y. Hong, "Modified Graded Repair of Cerebrospinal Fluid Leaks in Endoscopic Endonasal Transsphenoidal Surgery", *Journal of Korean Neurosurgical Society*, **58**(1), 36 (2015).
16. A. Tamašauskas, K. Šinkūnas, W. Draf, V. Deltuva, A. Matukevičius, D. Rastenytė, and S. Vaitkus, "Management of cerebrospinal fluid leak after surgical removal of pituitary adenomas", *Medicina*, **44**(4), 302 (2008).

17. C. Martín-Martín, G. Martínez-Capoccioni, R. Serramito-García, and F. Espinosa-Restrepo, "Surgical challenge: endoscopic repair of cerebrospinal fluid leak", *BMC Research Notes*, **5**(1), 459 (2012).
18. E. Sanders, R. Clark, and J. Katzmann, "Cerebrospinal Fluid Leakage: Agarose Gel Electrophoresis Detection of β 2-Transferrin and Nephelometric Quantification of β-Trace Protein", *Clinical Chemistry*, **50**(12), 2401 (2004).
19. T. Gorogh, P. Rudolph, J. Meyer, J. Werner, B. Lippert, and S. Maune, "Separation of β2-Transferrin by Denaturing Gel Electrophoresis to Detect Cerebrospinal Fluid in Ear and Nasal Fluids", *Clinical Chemistry*, **51**(9), 1704 (2005).
20. C. Papadea and R. Schlosser, "Rapid Method for β2-Transferrin in Cerebrospinal Fluid Leakage Using an Automated Immunofixation Electrophoresis System", *Clinical Chemistry*, **51**(2), 464 (2005).
21. C. McCudden, B. Senior, S. Hainsworth, W. Oliveira, L. Silverman, D. Bruns, and C. Hammett-Stabler, "Evaluation of high resolution gel β2-transferrin for detection of cerebrospinal fluid leak", *Clinical Chemistry and Laboratory Medicine*, **51**(2), (2013).
22. H. Wang, B. Kang, F. Ren, S. Pearton, J. Johnson, P. Rajagopal, J. Roberts, E. Piner, and K. Linthicum, "Electrical detection of kidney injury molecule-1 with AlGaN/GaN high electron mobility transistors", *Applied Physics Letters*, **91**(22), 222101 (2007).
23. B. S. Kang, H. T. Wang, T. P. Lele, F. Ren, S. J. Pearton, J. W. Johnson, P. Rajagopal, J. C. Roberts, E. L. Piner, and K. J. Linthicum, "Prostate Specific Antigen Detection using AlGaN/GaN High Electron Mobility Transistors", *Appl. Phys Lett.*, **91**, 112106 (2007).
24. B. Kang, S. Pearton, J. Chen, F. Ren, J. Johnson, R. Therrien, P. Rajagopal, J. Roberts, E. Piner, and K. Linthicum, "Electrical detection of deoxyribonucleic acid hybridization with AlGaN/GaN high electron mobility transistors", *Applied Physics Letters*, **89**(12), 122102 (2006).
25. J. Yang, P. Carey, F. Ren, Y. Wang, M. Good, S. Jang, M. Mastro, and S. Pearton, "Rapid detection of cardiac troponin I using antibody-immobilized gate-pulsed AlGaN/GaN high electron mobility transistor structures", *Applied Physics Letters*, **111**(20), 202104 (2017).
26. J. Yang, P. Carey, F. Ren, M. Mastro, K. Beers, S. Pearton, and I. Kravchenko, "Zika virus detection using antibody-immobilized disposable cover glass and AlGaN/GaN high electron mobility transistors", *Applied Physics Letters*, **113**(3), 032101 (2018).
27. C. Hsu, P. Chen, A. Pulikkathodi, Y. Hsiao, C. Chen, and Y. Wang, "A Package Technology for Miniaturized Field-Effect Transistor-Based Biosensors and the Sensor Array", *ECS Journal of Solid State Science and Technology*, **6**(5), Q63 (2017).
28. I. Sarangadharan, A. Regmi, Y. Chen, C. Hsu, P. Chen, W. Chang, G. Lee, J. Chyi, S. Shiesh, G. Lee, and Y. Wang, "High sensitivity cardiac troponin I detection in physiological environment using AlGaN/GaN High Electron Mobility Transistor (HEMT) Biosensors", *Biosensors and Bioelectronics*, **100**, 282 (2018).
29. Y. Wang, B. Chu, C. Chang, C. Lo, S. Pearton, A. Dabiran, P. Chow, and F. Ren, "Long-term stability study of botulinum toxin detection with AlGaN/GaN high electron mobility transistor based sensors", *Sensors and Actuators B: Chemical*, **146**(1), 349 (2010).
30. A. Kapoor, J. Ritter, and R. Yang, "An extended Langmuir model for adsorption of gas mixtures on heterogeneous surfaces", *Langmuir*, **6**(3), 660 (1989).
31. S. Stankowski, "Surface charging by large multivalent molecules. Extending the standard Gouy-Chapman treatment", *Biophysical Journal*, **60**(2), 341 (1991).
32. T. Heimburg and D. Marsh, "Protein surface-distribution and protein-protein interactions in the binding of peripheral proteins to charged lipid membranes", *Biophysical Journal*, **68**(2), 536 (1995).