Quantitative Electrolateral Flow Immunosensor (ELLI^{TM}): Opening new diagnostic frontiers such as Dengue Triage and Secondary Stroke Prognosis

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ABSTRACT

The development of a rapid, affordable, and sensitive diagnostic kit for point-of-care is important in most healthcare settings; however, only a few exhibit the needed quantitative capability. Our technology, ELLI^{TM}, is a patented diagnostic device that aims to provide on-site quantitation by utilizing proprietary electroactive immunonanoparticles that bind to the target biomarker and subsequently move along toward the biofunctionalized screen-printed gold electrodes (SPGE) to generate an amperometric signal. The SPGE functions simultaneously as a signal transducer and a solid-state support for a sandwich immunoassay. The successful immunocomplex formation is then recorded electrochemically using a potentiostat, whereby the signal was contributed by the presence of ferrocene or a more hydrophilic redox label, radical TEMPO, immobilized on the formulated nanoparticles. A bifunctional ligand, thiolated polyethylene glycol (PEG-thiol), was used to stabilize 20 nm gold colloidal nanoparticles (AuNPs). The ligand was incorporated to not only prevent the salt-mediated AuNPs aggregations but also provide an anchor for antibody and redox species conjugation. To-date, we have miniaturized a 3D-printed prototype device able to sensitively detect and quantify dengue NS1 protein with only 0.6 µ L human clinical serum samples diluted in a volume ratio of 1:100 in PBS diluent in less than 30 min with a simple cyclic voltammetry analysis. Other applications are being developed, including stroke prognosis using NT-proBNP biomarker, that is significantly associated with cardioembolic stroke and secondary stroke reoccurrence, with sensitivity >90% and specificity >80%. Initial results of this research in mock porcine plasma have shown that the label-free impedimetric electrochemical biosensor was capable of differentiating such concentrations in a low concentration range, especially among 0, 0.1, 0.5, 1, and 3 ng mL^{-1} within 25 min. This range is valuable not only for classifying cardioembolic stroke (>=0.5 ng mL^{-1}) but also predicting the risk of secondary stroke reoccurrence (>0.255 ng mL^{-1}). We are now adapting these results to the ELLI^{TM} platform. The applications above need quantitation as a measure of severity and a classification means. In dengue, for example, a simple, 1-2 step, point-of-care-format is recommended for disease surveillance in communities as a first line of defense, especially when there is no specific treatment for dengue/severe dengue. Meanwhile, in stroke prognosis, a quantitative point-of-care device that reduces the delays in IV-tPA administration and predicts risks as well as secondary reoccurrence will be highly favored. ELLI^{TM} promises to deliver sensitive measurements of the target biomarker and acts as a diagnostic intervention for faster therapeutics administration that eventually will contribute to a better disease emergency preparedness, such as in trauma triage (internal bleeding) for soldiers. ELLI^{TM} is the answer to whichever applications that would require quantitation of biomarkers or pathogens at the point of care such as for HAZMAT teams, the military, or home use.

Keywords: dengue triage, stroke prognosis, trauma triage, internal bleeding, quantitative diagnostics

Figure 1: Our capability in translational science extends to five different areas, each with different biomarkers of interest. ELLI^{TM} will provide quantification of biomarkers in different types of sample for diagnosis, monitoring, and outcome prediction.

1 WHY DO WE DO IT

In the last 50 years, dengue fever incidence has increased 30-fold, while the number of endemic countries has increased more than 4-fold and it continues to rise inexorably. No vaccines or specific antiviral medicines for dengue exist, except the newly approved and partially effective Dengvaxia® by Sanofi Pasteur, available for only in a few countries and now used with caution because of the phenomenon of antibody-dependent enhancement in the vaccinated seronegative population that outweighs the benefits (acting like a silent primary infection) [1]. Treatment solely relies on early diagnosis (especially for dengue hemorrhagic fever) and immediate supportive treatment to prevent death.

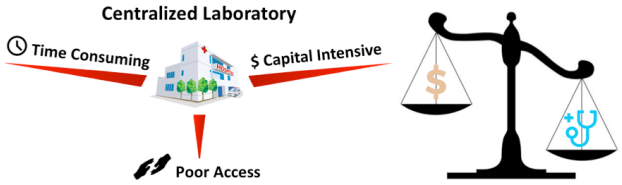

Figure 2: **Medical diagnostics problems:** They are mostly performed in a centralized lab; time-consuming, inaccessible, and expensive. There is no affordable way to measure the amount of disease biomarker at the point of care.

Presently, a number of commercial dengue diagnostic tests based on the qualitative detection of non-structural 1 (NS1) protein are being used in point-of-care testings. The flavivirus NS1 protein has been recognized as an important immunogen in infection, particularly in dengue triage because this protein is secreted in the bloodstream and can be detected on day 1 after the onset of fever while a threshold level is indicative of a putative severe case. One should note that although the rapid lateral flow strip has been fittingly satisfactory providing qualitative diagnosis, there is an exigency of NS1 protein point-of-care quantitation in dengue prognosis of the disease severity. Individuals are more prone to severe cases when the number of circulating NS1 protein is higher, notwithstanding the fact that the infecting serotype and patients' immune status are equally pivotal [2, 3].

Meanwhile, stroke has affected around 15 million people each year, occurring in an average of 214 out of 100,000 people every year in developed nations. In the U.S., a new case of stroke happens every 40 seconds, or approximately 2,200 people every day.

Stroke diagnosis is done via various techniques and imaging tools, such as CT/MRI scan. However, current clinical guidelines emphasize the need for timely and early stroke care, such as with the use of NT-proBNP biomarker for diagnosis of heart failure and now, characterization of cardioembolic stroke. Quantitative measurement of biomarkers provides more accurate and complete information regarding disease progression or response to a specific drug therapy; they are essentially used for diagnosing and predicting the disease outcome. Even though there are commercial kits sufficiently provide quantitative results, the application is limited to the use of expensive optical readers (upward of USD 1,000) hence making them impractical for a widespread use as a point-of-care technology, particularly in less developed healthcare settings (Fig. 2).

Increased NT-proBNP levels are indicative of cardioembolic stroke with a sensitivity of 75.6% and specificity of 87.4%. Furthermore, elevated NT-proBNP levels have also been shown to predict the reoccurrence of secondary stroke, whereby levels greater than 255 pg mL^{-1} pose a significantly higher risk with a sensitivity of 76% and specificity of 60% [4].

In both cases of dengue and stroke, it is evident that providing the quantitative measurement of biomarkers at the point of care is critically important (Fig. 2). Therefore, research should be focused on developing a quantitative, yet affordable, rapid, portable, and user-friendly biosensor so as to be in accordance with ASSURED diagnostics rapid test criteria by WHO.

In search of that, electrochemical sensors are the most promising tool to provide an attractive quantitation means to analyze a biological sample and the possibility of system miniaturization so as to be cheap, small, portable, and user-friendly, epitomizing the glucose sensor. Additionally, paper-based microfluidics have become popular because of their low cost, ease of fabrication, disposability, and convenience of liquid transport without applying an external driving force. For these two reasons, there is great interest in combining electrochemistry and paper microfluidics for point-of-care diagnostics (Fig.3), and with the advancement of technology, a digital quantitation is soon going to be feasible with the incorporation of a mobile reader to a lateral flow strip [5].

Biosensorix is developing a universal quantitative platform to fulfill the need for biomarkers measurement in bodily fluids. Our technology, ELLI^{TM}, measures the electrochemical signal generated by our proprietary immunoformulation that contains redox species-tagged AuNPs flowing to the SPGE forming sandwich-like immunoassay [2, 3]. ELLI^{TM} intents to provide sensitive measurements of the target biomarker and perform as a diagnostic intervention for disease triage and faster therapeutics administration: saving lives from dengue and preventing stroke at home. In addition to two cases mentioned above, ELLI^{TM} can be a promising technology in trauma triage such as in the case of internal bleeding in soldiers. ELLI^{TM} envisions to be the answer to whichever applications that would require quantitation of biomarkers or pathogens at the point of care.

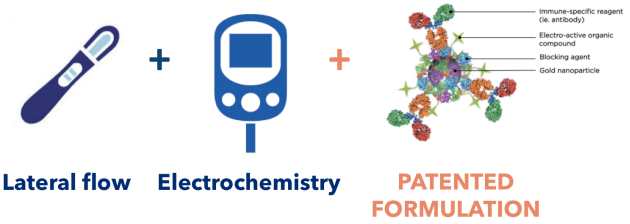

Figure 3: **Quantitative medical diagnostics solution**: Combining the two most well-defined point-of-care technologies, lateral flow and electrochemistry, and adding our patented formulation, is a formula for affordable and accessible quantitative diagnostics.

2 HOW DO WE DO IT

As described in our previous work [2], there are four major components in the construction of ELLI^{TM} for dengue: (1) formulation of our proprietary electroactive immunonanoparticles, (2) biofunctionalization of SPGE with antibody, (3) paper membrane preparation with the AuNPs conjugates and the appropriate buffer, and (4) 3D-printed housing. All of them were then assembled and enclosed in the 3D-printed housing as shown in Fig. 4.

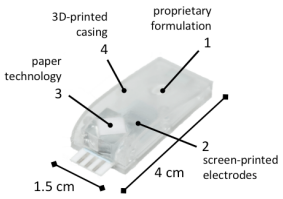

Figure 4: ELLI^{TM} is a thumb-size miniaturized electrolateral flow that provides a quantitative measurement of a target analyte.

2.1 ELLI^{TM} for dengue with NS1 protein

The immunoassay rationale is illustrated in Fig 5. Briefly, prior to the immunoassay, dengue NS1 protein was serially diluted in PBS buffer to achieve various concentrations from 1000 ng mL^{-1} to 50 ng mL^{-1}. With the ELLI^{TM} strip ready, the NS1 protein sample was applied at the sample window, followed by the running buffer. The electroactive immunonanoparticles sitting on the conjugate pad formed immunocomplexes with the sample NS1 protein and flowed toward the biofunctionalized SPGE where they were captured and immobilized. The process took 20 min before the housing was tilted axially to ensure the flow of unspecific complexes away from the SPGE to the absorbent pads. After 2 min of the tilted flow, the strip was connected to the potentiostat via the connector. Cyclic voltammetry (CV) was run and then, the values of the anodic peak were recorded.

In a further study, six clinical samples were tested with ELLI^{TM}. Using a similar procedure to the buffered samples, the 0.6 µL human serum clinical analyte diluted in 1:100 with PBS diluent was introduced at the sample window and followed by the running buffer. Afterward, a cyclic voltammogram of each sample was obtained to be analyzed in order to generate the anodic peak value. The average was then plotted.

2.2 ELLI^{TM} for stroke with NT-proBNP

After a preliminary assessment [4], NT-proBNP was tested further in ELLI^{TM}. Similar to the dengue assay, NT-proBNP was serially diluted in PBS buffer to various concentrations from 5 ng mL^{-1} to 0.1 ng mL^{-1}. After 20-min incubation of 60 µL NT-proBNP protein and 90 µL running buffer, the housing was tilted axially and another 90 µL drop of running buffer was introduced. Afterward, the strip was connected to the potentiostat via the connector and the cyclic voltammograms were recorded. Then, the values of the anodic peak were recorded

3 WHAT HAVE WE DONE

3.1 ELLI^{TM} successfully quantified dengue NS1 protein in buffer and clinical samples

As reported earlier [2], ELLI^{TM} is a miniaturized electrolateral flow prototype developed to detect dengue NS1 protein by a sandwich immunoassay involving the

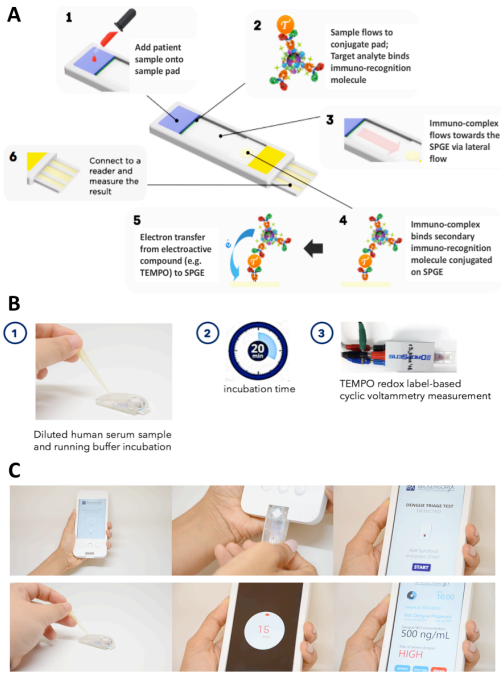

Figure 5: (A) The assay rationale of ELLI^{TM}. *(Reproduced with permission)* (B) ELLI^{TM} is as an amperometric point-of-care testing for biomarkers measurement in less than 30 min. Results can be obtained after 20 min incubation using a potentiostat to measure the electrochemical signals associated with specific recognition in positive samples. (C) Various field applications are soon possible with a handheld reader and a user-friendly interface to provide results in 15 min.

detection dengue NS1 antibodies-modified nanoparticles and a capture dengue NS1 antibodies-functionalized microelectrode. CV was performed to investigate the signal of the TEMPO• label tagged to the formulated immunonanoparticles bound over the SPGE surface. It should be noted that the capture and detection antibodies are elicited against a different NS1 epitope, thus sandwich formation is possible. A calibration plot was obtained from the average anodic peak value of each NS1 protein concentration in PBS with the linear range between 100 and 1000 ng mL^{-1} (Fig. 6A). ELLI^{TM} was able to detect dengue NS1 protein down to 50 ng mL^{-1}; the limit of detection was calculated using the blank signal plus three standard deviations.

After generating a calibration curve, the clinical samples were tested with ELLI^{TM}. The average anodic peak value was then plotted and analyzed (Fig. 6B). the cut-off point was determined to be just below 1 µA (approx. 0.8 µA) based on the three negative samples. Despite being higher than the blank, samples 1–3 were considered negative because their values were below the three standard deviations of the blank average. Therefore, three other samples (4–6) were considered positive and the value of NS1 protein could be estimated by a back-calculation using the equation from the previously obtained calibration plot (Fig. 6A). From this, the NS1 protein concentration in diluted clinical samples was estimated to be 51.04 ng mL^{-1}, 107.73 ng mL^{-1}, and 305.08 ng mL^{-1} for sample 4, 5, and 6, respectively.

3.2 Optimization of ELLI™ for stroke with NT-proBNP

Following the promising results of ELLI™ with dengue and the NT-proBNP's relevant clinical performance of our

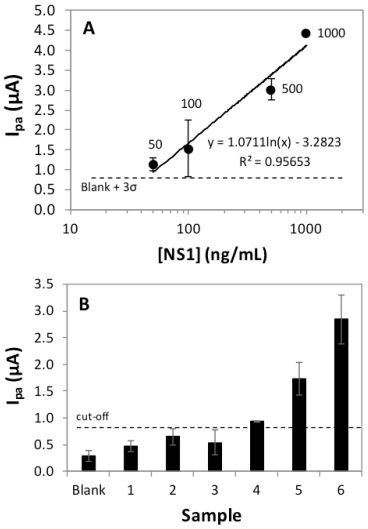

Figure 6: (A) A calibration plot of dengue NS1 protein was generated for ELLI™. (B) Data charts of the measured anodic peak signals of six diluted clinical samples by ELLI™, each evaluated twice in separate prototypes. The blank value (i.e. PBS only) was an average of four separate measurements. *(Reproduced with permission)*

electrochemical biosensor, further NT-proBNP tests were conducted to validate the capability of ELLI™ as a universal platform (Fig. 7). Although there was yet a clear proportional correlation, ELLI™ platform has shown a favorable trend in increasing signals with a concurrent increase of NT-proBNP concentrations. To-date, ELLI™ is seen to be able to detect NT-proBNP in buffer down to 0.1 ng mL^{-1}. Future studies are to be carried out in order to improve the sensitivity and establish parameters for fabricating a universal platform enabling the ease of substitution of target analytes.

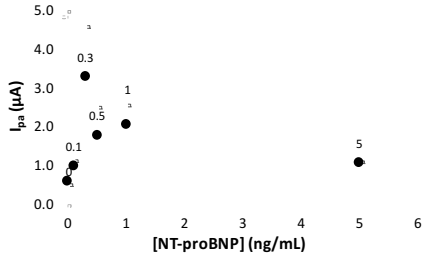

Figure 7: A calibration plot of NT-proBNP in ELLI™ was generated for concentrations from 5 ng mL^{-1} down to 0.1 ng mL^{-1}.

4 HOW DO WE DIFFER

ELLI™ prototype shows excellent performance toward detection of analyte dengue NS1 protein and exhibits a fairly low limit of detection suitable for acute samples. The development of ELLI™ which combines electrochemistry and paper microfluidics provides an affordable and simple technology thus potentially offering on-site point-of-care testings, delivered along with a mini potentiostat to epidemic sites or the point of care enabling a fast-on-site quantitative detection of dengue and stroke analytes. ELLI™ envisages not only elimination of the impractical storage and handling of the probes and reagents that are commonly found in optical sensors, but also significant cost reduction of the reader. Moreover, we have presented a miniaturized biosensing platform for detecting dengue NS1 protein in infected clinical serum samples with good sensitivity. The assay is fully integrated, compatible with a fingerprick sample volume, and the result is relatively fast (less than 30 minutes). Preliminary results on NT-proBNP measurement showed promising future developments. Therefore, it is safe to say that ELLI™ has the potential to act as a rapid quantification diagnostic point-of-care kit not only for dengue but also other diseases that need quantitation such as stroke at home and trauma injury, particularly internal bleeding for soldiers.

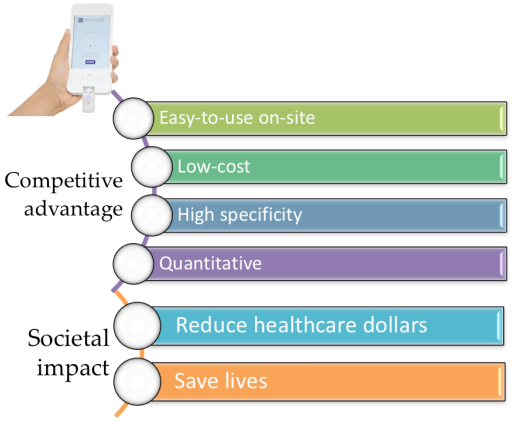

Figure 8: What the future of diagnostics will bring. Biosensorix's ELLI™ promises to bring access to a quantitative platform that is sensitive, rapid, user-friendly, and low-cost so as to save lives and resources. We can speed up dengue and stroke prognosis leading to a faster treatment. The goal is also to prevent stroke at home.

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