An ultrasensitive and culture-free detection platform for microorganisms and protein biomarkers

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Keywords: Ultrasensitive detection, rapid detection, biomarker detection, bacteria detection, early detection, culture free detection

Abstract

We have developed an electrochemical immunoassay platform, which detects a range of disease-related targets including bacteria, viruses, fungi and protein biomarkers. The platform features a voltage-controlled signal amplification, which leads to ultrasensitive detection. The analytical performance of the platform has been validated with protein biomarkers and bacteria. The detected biomarkers include CA 125, PSA in serum and AMACR, a novel biomarker for prostate cancer, in serum and urine. The PSA and AMACR detections were performed on the femto gram/mL level. S100 B, a biomarker for traumatic brain injury, was detected in serum with a detection limit of 10 fg/mL level. Because of its ultrasensitivity, the platform detects bacteria directly in urine and whole blood without sample culture. We have detected *E. coli*, *K. pneumonia*, *P. aeruginosa*, *N. gonorrhroae, S. aureus, MRSA* and *S. pneumoniae* in prepared and clinical samples. The current detection limit for bacteria is 3 CFU/mL in blood. We have demonstrated the platform's capacity as a diagnostic technology for bacterial infections. The platform showed a detection-identification time of 84 minutes, and a time-to-result for antibiotics susceptibility testing of 204 minutes. We have conducted an independent (3rd party) validation of the platform.

1 Introduction

State-of-the-art technologies for the diagnosis of bacterial infections, which consists of the detection-identification-antibiotics susceptibility testing (AST) steps, are based on fluorescence, mass spectrometry or polymerase chain reaction. These technologies require positive sample culture, leading to a time-to-result of 18-72 hours. The long diagnosis time due to culture results in the use of broad-spectrum antibiotics, which results in under-treatment, harmful side effects and the prevalence of drug resistant microorganisms. The detection of protein biomarkers using enzyme-linked immunosorbent assay is known to provide unsatisfactory sensitivity. We have developed a novel electrochemical immunoassay technique [1], which features an intrinsic amplification of the signal current controlled by an external gating voltage. The patented technique was used to obtain a detection limit for molecular analytes on the zepto-molar (10^{-21}M) level [2]. We have developed this technique to establish a detection platform. The platform detects a range of disease-related targets including bacteria, viruses, fungi and protein biomarkers. We have demonstrated the ultrasensitivity of the platform with bacteria and biomarkers. Here, we show that the ultrasensitivity leads to culture-free detection-analysis of bacteria in blood and early detection of diseases. The platform was realized with screen printed electrodes (SPEs), intended for point-of-care, disposable and low-cost applications.

2 The detection platform

We have invented field-effect enzymatic detection (FEED)[1], a novel bio-sensing technique, in which an external gating voltage V_G is used to provide intrinsic amplification of the signal current of an enzymatic biosensor. Figure 1(a) shows the detection setup. A three-electrode electrochemical system is modified with the gating electrodes, which applied the gating voltage V_G to the redox enzyme (green elliptical structures) immobilized on the working electrode. V_G induces ions at the solution-electrode interface to set up an electric field within the enzyme to modulate interfacial charge transfer. The field lowers the tunnel barrier between the electrode and the active center of the enzyme and therefore increases the tunnel current to provide amplification to the signal. The patented quantum mechanics-based technique was used to obtain the detection limit of molecular analytes on the zepto-molar (10^{-21}M) level[2].

We have incorporated FEED with the immunoassay approach to establish a novel detection platform. Figure 1(b) shows the antibody-antigen-antibody immune complex formed on the working electrode of the FEED system. The enzyme used to label the detecting antibody is immobilized on the electrode via the complex. FEED provides ultrasensitivity due to its intrinsic amplification whereas the immunosensing technique provides a high degree of substance selectivity. The detection system was realized on SPEs, intended for point-of-care, disposable and low-cost applications. Figure 1 (c) shows the voltage controlled amplification of the detection signal of 8 CFU/mL *E. coli*.

3 Bacteria detection-identification-AST

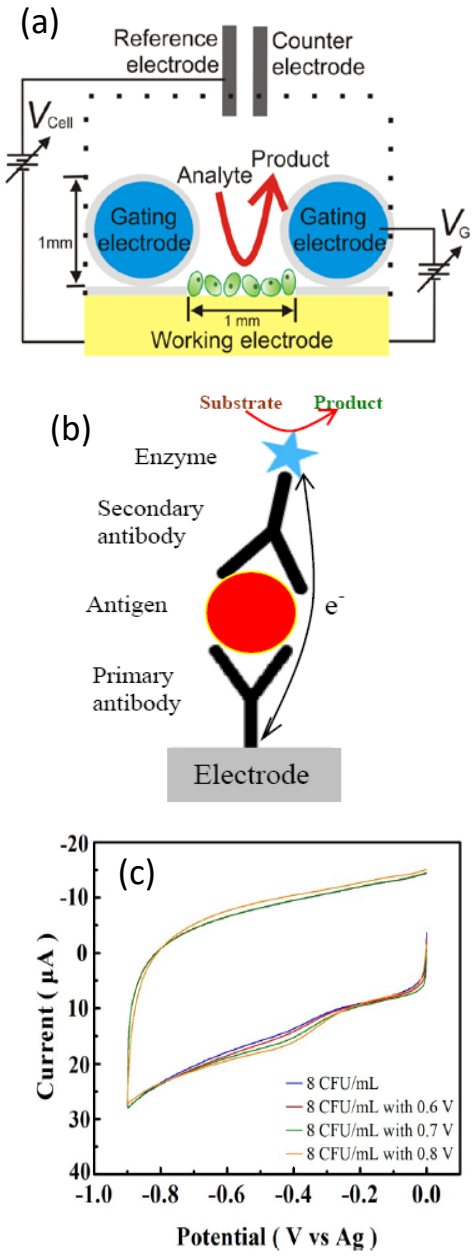

Figure 1 (a) FEED setup. (b) Sandwich immune complex formed on the working electrode of the platform. (c) Amplification of the signal of the detection of 8 CFU/mL *E. coli*. The reduction peak (-0.4 V) of the HRP used to label the detection antibody is the signal. Figure 3 (c) is taken from reference [3].

We have used the platform to perform detection-identification (ID)-antibiotics susceptibility testing (AST) of *E. coli* spiked in human blood [3]. WT *E. coli* (ACTT 25922)) was transformed to an ampicillin-resistant (ampR) strain to provide a model for AST. The rate of successful detection in bacteria-spiked samples (positive control) is 98% (based on 200 measurements). The rate of detecting the absence of the signal in negative controls is 98% (based on 50 measurements). The results were authenticated by traditional culture with colony enumeration. The limit of detection was 3 CFU/mL.

Simultaneous detection and ID

Since the selectivity of the platform is based on the specific immune interaction between a bacterium and its antibody, the platform allows the detection of bacteria and their ID in a sample to be performed simultaneously. Figure 2 (a) shows a paradigm of this methodology developed for a two-bacteria test case. Figure 2 (b) shows the feasibility of this paradigm using a blood sample containing *E. coli* and *L. innocua* (ATCC 33090) and the corresponding bacterium specific detection electrodes. Blood samples spiked with each or both bacterial species were used. The bacterial concentration was confirmed by culture (green numerical values). The platform detection and culture results for 11 CFU/mL *E. coli* and 45 CFU/mL

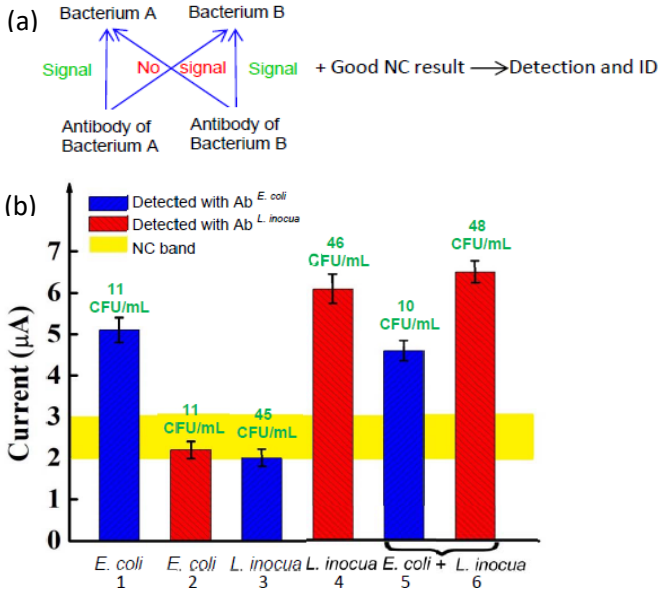

Figure 2 (a) Paradigm for simultaneous detection and ID for the two-species test case (b) Example results for this paradigm. Figures are taken from reference [3].

L. innocua are shown in Figure 2 (b). The process was completed in 84 minutes at 25 °C. The detection of negative control samples (blood samples not spiked with bacteria) defines a negative control (NC) band (the yellow band), whose range covers 2-3 μA.

In the *E. coli*-only samples (Bar 1 and Bar 2), a signal is detected above the NC band using the *E. coli* specific electrode but not the *L. innocua* specific electrode. This indicates the platform's selectivity for *E. coli* against *L. innocua*. Likewise, only the *L. innocua* specific electrode detects a signal above the NC band for the *L. innocua*-only samples (Bar 3 and Bar 4), demonstrating selectivity for *L. innocua* against *E. coli*. In samples containing both *E. coli* and *L. innocua*, both types of electrodes detect a signal. Using an *E. coli* specific electrode, a signal of 4.7 μA was measured from a sample containing nominally 10

CFU/mL *E. coli* and 40 CFU/mL *L. innocua* whereas culture indicates that there are 10 CFU/mL of *E. coli* in the (Bar 5). Using an *L. innocua* detection electrode yield a signal of 6.4 mA whereas culture shows 48 CFU/mL in the sample (Bar 6). Note that these signals are very close to the signals from samples that contain only one bacterial species. This indicates negligible detection interference between the two species in these samples. Therefore, the results in Figure 2 (b) indicate that *E. coli* and *L. innocua* were quantitatively detected and identified simultaneously.

AST

Wild type (WT) *E. coli* and ampR *E. coli* were used to demonstrate the AST capacity of the platform as shown in Figure 3. The *E. coli* strains were spiked individually in human blood at 8 CFU/mL. The platform is able to provide AST on bacterial samples that have been exposed to antibiotics for 1-2 hours. Figure 3 shows the growth

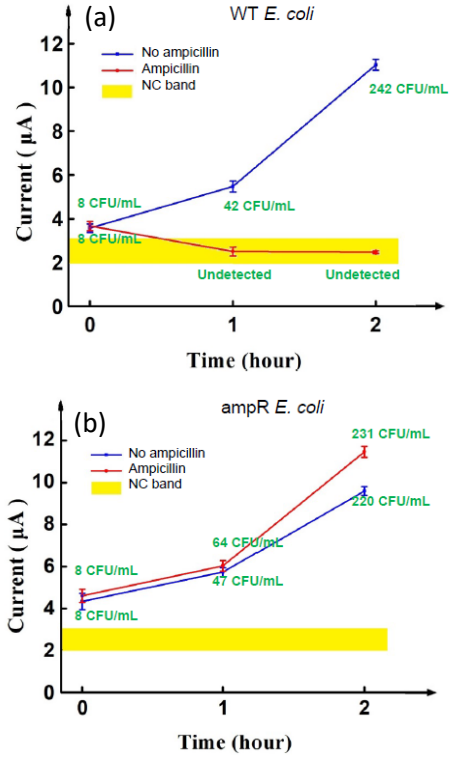

Figure 3 Platform based AST of (a) WT *E. coli*, (b) ampR *E. coli*. Figures are taken from reference [3].

responses of the two strains to 8 μg/mL ampicillin (The minimum inhibitory concentration of ampicillin for the WT strain was 3 μg/mL) detected using the platform. When exposed to ampicillin, the concentration of WT. *E. coli*, which is sensitive to ampicillin, becomes undetected (in the NC band, Figure 3 (a)), while ampR *E. coli* shows continuous growth in the presence of ampicillin (Figure 3 (b)). The AST was completed in 204 minutes (120 min exposure to ampicillin + 84 min for detection). The detected bacterial growth is confirmed using culture (green numerical values). The results also indicate that even at 1-hour, the two strains can be differentiated, leading to 144 min AST.

We have performed an analytical validation of the platform following the Clinical Laboratory Improvement Amendments (CLIA) format developed for the validation of laboratory-developed assays for infectious diseases [4]. The validation characterized the properties of the platform, including linear response range, analytical sensitivity and detection limit, analytical specificity (inclusivity and exclusivity), precision and accuracy and test sensitivity (false positive /false negative).

Recent developments on the platform include testing with clinical urine and blood samples and a 3rd party validation.

4 Detection of biomarkers

The flexibility of the platform is reflected in its detection of protein biomarkers. The detected cancer biomarkers include cancer antigen 125 (CA125, a biomarker of ovarian cancer) [5], prostate specific antigen (PSA, a biomarker of prostate cancer) in serum [6] and alpha-methylacyl-CoA racemase (AMACR, a novel marker of prostate cancer) in serum and urine [7]. The PSA and AMACR detections were performed on the pico-femto gram/mL level. Most recently, we have achieved the detection of S100 B, a biomarker for mild traumatic brain injury (mTBI), in serum on the 10 fg/mL level [8]. The calibration curves cover four orders of magnitudes from 10 fg/mL to 10 ng/mL. Figure 4 shows the calibration curve in the fg/mL range. The specificity of the detection was demonstrated using TAU protein, which is another marker for mTBI.

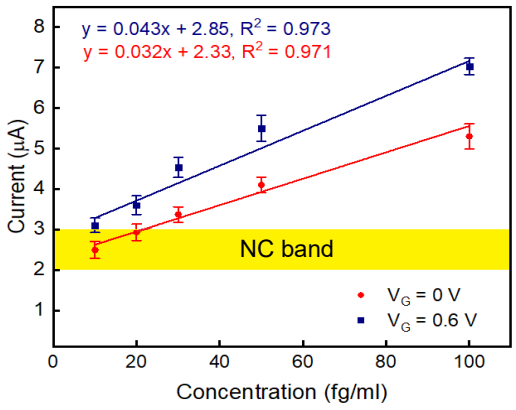

Figure 4 The S100B calibration curve on the fg/mL level. NC band stands for negative control band. Figure is taken from reference [8].

Our results indicate the feasibility of using this platform for the detection of extremely low concentrations of markers of mTBI in human serum and possibly in other body fluids. The ultrasensitive platform will be able to detect clinically validated biomarkers that carry diagnostic and prognostic information at extremely low

levels in body fluids. In fact, the demonstrated fg/mL level detection will allow for the detection of the biomarkers at the onset of their leaking from cerebrospinal fluid into the blood stream via a compromised blood–brain barrier [9], making the platform a truly early-diagnosis technology for mTBI. The platform can also be used as a research instrument to discover novel TBI biomarkers that exist in extremely low concentrations in body fluids that cannot be detected using ELISA or other methods. Further, the platform can be used to assess the effect of future pharmacological treatments by detecting minute amounts of molecular markers/indicators.

5 Innovation and significance of platform

The innovative nature of the platform is reflected in that it will perform all three steps of infection diagnosis within a timeframe that is dramatically shorter than that of the standard of care. This new diagnostic approach can be easily adopted into the routine clinical laboratory setting due to its compact format and ease-of-use. Therefore, the platform has the potential to replace the current infection diagnostics and lead to a fundamental change in the clinical practice for infections.

The realization of the platform will lead to fundamental changes in the diagnosis of bacterial infections. The significantly shortened detection-ID-AST time provided by the platform will allow clinicians to use at most one dose of broad-spectrum antibiotics in the beginning of treatment (required by clinical guidelines) and start using targeted treatment based on narrow spectrum antibiotics for the subsequent doses of antibiotics. The fact that the platform will ultimately reveal species ID in 70 minutes will allow clinicians to narrow empiric antibiotics based on species alone even before the first dose, leading to lowered cost of treatment, reduced side effects and decreased emergence resistance. The change in the paradigm of treatment will result in significantly improved efficacy and reduced detrimental side effects. The limited use of broad-spectrum antibiotics will effectively reduce the prevalence of multiple drug resistant organisms. The realization of the proposed platform will lead to more effective stewardship of antibiotics. Therefore, the platform will be a potential game-changer.

Another area of application for the platform is food safety. Previously, we used the platform to detect *E. coli* O157:H7 in milk and meat juice and *Shigella* in stool. [10-12]. The infectious doses (ID) of bacteria are usually low, e.g. 10 cells. Since the platform is able to detect extremely small amounts (<10 cells) of bacteria directly in complex matrices, it will enhance the analysis of food samples by providing ultrasensitivity and rapid time-to-result. In general, the detection platform provides three distinctive advantages: (1) ultrasensitive detection of bacteria and biomarkers in complex matrices, (2) real-time/rapid detection of bacteria without the culture process, and (3) low-cost detection.

5 Conclusion

The results presented here indicate that the FEED-based immuno-assay platform is a versatile platform for the ultrasensitive, rapid detection of biological antigens. Its electrochemical nature allows the platform to be used in low-cost, disposable and POC applications.

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