Biosensors and Bioelectronics 26 (2010) 649–654

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Electrochemical immunosensor detection of urinary lactoferrin in clinical samples for urinary tract infection diagnosis

Ying Pan[a], Geoffrey A. Sonn[a], Mandy L.Y. Sin[b], Kathleen E. Mach[a], Mei-Chiung Shih[c,d], Vincent Gau[e], Pak Kin Wong[b], Joseph C. Liao[a,f,*]

[a] Department of Urology, Stanford University, Stanford, CA, USA
[b] Department of Aerospace and Mechanical Engineering, The University of Arizona, Tucson, AZ, USA
[c] Department of Health Research and Policy, Stanford University, Stanford, CA, USA
[d] Veterans Affairs Cooperative Studies Program Coordinating Center, Mountain View, CA, USA
[e] GeneFluidics, Inc., Monterey Park, CA, USA
[f] Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA

ARTICLE INFO

Article history:
Received 4 April 2010
Received in revised form 1 July 2010
Accepted 2 July 2010
Available online 8 July 2010

Keywords:
Electrochemical biosensor
Amperometry
Urinary diagnostics
Urinary tract infections
Biomarkers

ABSTRACT

Urine is the most abundant and easily accessible of all body fluids and provides an ideal route for non-invasive diagnosis of human diseases, particularly of the urinary tract. Electrochemical biosensors are well suited for urinary diagnostics due to their excellent sensitivity, low-cost, and ability to detect a wide variety of target molecules including nucleic acids and protein biomarkers. We report the development of an electrochemical immunosensor for direct detection of the urinary tract infection (UTI) biomarker lactoferrin from infected clinical samples. An electrochemical biosensor array with alkanethiolate self-assembled monolayer (SAM) was used. Electrochemical impedance spectroscopy was used to characterize the mixed SAM, consisted of 11-mercaptoundecanoic acid and 6-mercapto-1-hexanol. A sandwich amperometric immunoassay was developed for detection of lactoferrin from urine, with a detection limit of 145 pg/ml. We validated lactoferrin as a biomarker of pyuria (presence of white blood cells in urine), an important hallmark of UTI, in 111 patient-derived urine samples. Finally, we demonstrated multiplex detection of urinary pathogens and lactoferrin through simultaneous detection of bacterial nucleic acid (16S rRNA) and host immune response protein (lactoferrin) on a single sensor array. Our results represent first integrated sensor platform capable of quantitative pathogen identification and measurement of host immune response, potentially providing clinical diagnosis that is not only more expeditious but also more informative than the current standard.

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1. Introduction

Urinary tract infection (UTI) is among the most common bacterial infections with an annual healthcare expenditure of $3.5 billion in the United States (Freedman, 2005; Griebling, 2005a,b). While *Escherichia coli* is the most common pathogen, UTI can also be caused by a variety of other pathogens, including *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus*. Similar to majority of other bacterial infections, diagnosis of UTI depends on bacterial culture, which has a significant time delay. One of the shortcomings of standard UTI diagnosis is that it does not define the infection severity, which would be helpful to guide antibiotic treatment. For example, the treatment of asymptomatic bacteriuria, lower urinary tract (i.e. bladder) infection, and upper urinary tract (i.e. kidney) infection are significantly different, ranging from no treatment, 3 days of antibiotics, to 7–14 days of antibiotics, respectively (Schaeffer and Schaeffer, 2007). A diagnostic platform capable of rapid determination of infection severity, therefore, would be of great clinical importance.

Electrochemical biosensors are molecular sensing devices that couple a biological recognition element to an electrode transducer (Drummond et al., 2003; Wang, 2005, 2006). Due to their excellent sensitivity, ease of miniaturization, and low-cost, electrochemical biosensors are well suited for point-of-care applications. A useful strategy to modify the sensor surface is self-assembled monolayer (SAM) (Baldrich and Laczka, 2008; Ulman, 1996; Wink, 1997). Consisted of regularly oriented, single layer of bifunctional organic molecules, SAM is thought to block adsorption of non-specific molecules to the sensor surface and thereby improve the signal-to-noise ratio (SNR). SAM can be either homogeneous or mixed,

* Corresponding author at: Department of Urology, 300 Pasteur Drive S-287, Stanford University, Stanford, CA 94305-5118, USA. Tel.: +1 650 852 3284; fax: +1 650 849 0319.
E-mail address: jliao@stanford.edu (J.C. Liao).

0956-5663/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.bios.2010.07.002

and tailored with different terminal functional groups to detect a wide variety of target molecules. While SAM-based electrochemical biosensors have been well described, they have primarily focused on detection of synthetic targets and purified samples (Chaki and Vijayamohanan, 2002). A potential advantage of SAM-based electrochemical biosensors is the reduction of matrix effects – particularly in clinical samples – on overall detection SNR, although this not well characterized (Chiu et al., 2010).

We recently described rapid multiplex detection of bacterial pathogens directly from clinical urine samples using an amperometric biosensor coated with an alkanethiolate (11-mercaptoundecanoic acid, MUDA) SAM (Liao et al., 2006, 2007; Mach et al., 2009). The detection strategy is consisted of sandwich hybridization between a pair of short DNA capture and detector probes to bacterial 16S rRNA, followed by signal amplification with horseradish peroxidase (HRP). The biosensor assay can deliver sample-to-answer within 1 h, a marked improvement from 2 to 3 days required for standard bacterial culture. Similar to bacterial culture, however, the biosensor assay does not provide information regarding infection severity to better guide treatment. A biosensor assay capable of simultaneously detect the offending pathogen while quantifying the host immune response will significantly improve the diagnosis of UTI and other infectious diseases.

In this report, we describe the development of an electrochemical immunoassay optimized to detect urinary biomarkers from infected clinical urine samples. We hypothesize that urinary lactoferrin (LTF), a 80 kDa iron-binding protein secreted by polymorphonuclear white blood cells (WBC), is a useful target for electrochemical immunoassay development as well as a predictive UTI biomarker as the average concentration of LTF was found to be 30.4 ng/ml in health urine and 3300 ng/ml in infected urines (Arao et al., 1999; Farnaud and Evans, 2003; Ward et al., 2005). We determined the optimal SAM configuration and capture/detector antibody combination to maximize the amperometric signal while avoiding non-specific bindings. This was followed by analytical and clinical validation of LTF detection to determine the limit of detection (LOD) and assay compatibility in a large number of clinical samples. We correlated urinary LTF level to the presence of WBC in urine (pyuria)—an important hallmark of UTI. Finally, we demonstrated pilot multiplex detection of urinary pathogens and LTF in clinical samples through simultaneous nucleic acid (i.e. 16S rRNA) and protein (i.e. LTF) assay on a single sensor array. Taken together, we have established a versatile biosensor platform for quantitative detection of an infection biomarker in clinical samples.

2. Experimental

2.1. Reagents

Potassium ferricyanide ($K_3Fe(CN)_6$), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), ethanolamine, bovine serum albumin (BSA), sodium acetate, casein from bovine serum, streptavidin and human LTF were purchased from Sigma–Aldrich (St. Louis, MO). EZ-link® Amine-PEG_2-Biotin was purchased from Pierce (Rockford, IL). Rabbit biotinylated polyclonal anti-LTF (ab25811), rabbit polyclonal anti-LTF (ab15811), mouse monoclonal anti-LTF (ab10110), rabbit horseradish peroxidase (HRP)-conjugated polyclonal anti-LTF (ab24264) and fluorescein isothiocyanate (FITC)-conjugated polyclonal anti-LTF were purchased from Abcam (England). Anti-fluorescein-peroxidase (Fab fragments) was purchased from Roche Diagnostics (Mannheil, Germany). Substrate solution for HRP (K-Blue Aqueous TMB) was purchased from Neogen (Lexington, KY).

The biosensors were washed with deionized (DI) water purified using a Millipore MilliQ A10 system (Bedford, MA). Phosphate buffered saline (PBS, pH 7.2) was purchased from Fisher Scientific (Fair Lawn, NJ) and consisted of 11.9 mM phosphates, 137 mM NaCl, and 2.7 mM KCl. PBS with 0.1% BSA was used to dilute the capture antibody, LTF standard, and urine samples. The detector antibody and HRP-conjugated goat anti-rabbit antibody were diluted with PBS containing 0.5% BSA. EZ-link® Amine-PEG_2-Biotin was diluted with 50 mM acetate buffer (pH 5.5) and streptavidin was diluted with DI water. The biosensors were blocked with PBS containing 0.5% (W/V) casein, then by 1 M ethanolamine (pH 8.5).

2.2. Apparatus

Electrochemical impedance spectroscopy was performed with a LF impedance analyzer (HP, 4192A). The electrochemical measurements were carried out using a 16-channel potentiostat (GeneFluidics, Monterey Park, CA) controlled with GF Reader 1.1.0.44 software. Sensor chips containing an array of 16 sensors were obtained from GeneFluidics. Each sensor is composed of three planar gold electrodes: working, reference, and counter (Gau et al., 2005; Liao et al., 2006). For the ELISA, optical absorbance at 450 nm was measured using a 96-well plate reader and the associated software Gen 5 (BioTek Instruments Inc., Winooski, VT).

2.3. Impedance analysis of self-assembled monolayer (SAM)

Electrochemical impedance spectroscopy was performed to evaluate the property of the SAM coating. A laser-machined plastic well manifold was first bonded to the sensor array for liquid containment. Wells were then filled with 50 μl of 1× PBS solution (Sigma, P5493) as a conductive working buffer. Magnitude and phase were measured at an oscillating voltage of 0.1 V to ensure linear response of the system. The impedances of the SAM layers were scanned with a frequency range from 5 Hz to 13 MHz. The results represent the mean of three measurements obtained with different sensors while the error bars represent the standard error. All experiments were performed at room temperature.

2.4. Biosensor surface preparation

All biosensor electrode surfaces were coated with either a SAM of 11-mercaptoundecanoic acid (MUDA) or a mixed SAM of MUDA and 6-mercapto-1-hexanol (MHOH). To prepare the mixed SAM, MUDA and MHOH were mixed 1:5 in a plasma-cleaned glass container and agitated with magnetic stirrer. The bare gold sensor chips were incubated in the mixture for 2 h followed by rinsing with ethanol and drying with nitrogen. The dried chips were placed in nitrogen cabinet at least 3 days prior to assay. The SAM on the sensor surface was activated by 4 μl of a 1:1 mixture of 100 mM NHS and 400 mM EDC on the working electrode, followed by 4 μl of 5 mg/ml EZ-link® Amine-PEG_2-Biotin. 4 μl of 0.5% casein in PBS and 30 μl of 1 M ethanolamine were then loaded on the sensor electrode sequentially to block the remaining EDC/NHS activated carboxyl groups on the biosensor surface, followed by 4 μl of 0.5 mg/ml streptavidin. Each incubation step lasted 10 min and was done at room temperature. Finally, 4 μl of diluted capture antibody was added to each working electrode and incubated for 1 h at 37 °C, completing the surface preparation (Fig. 1A).

2.5. Electrochemical biosensor assay protocol

Purified human LTF or clinical urine samples (see below) were added to the sensor working electrodes functionalized with the capture antibody. Four different urine dilutions were tested (undiluted, 5-, 20-, and 100-fold dilution). Samples were tested in duplicate. After 1 h incubation at 37 °C, 4 μl of HRP-conjugated

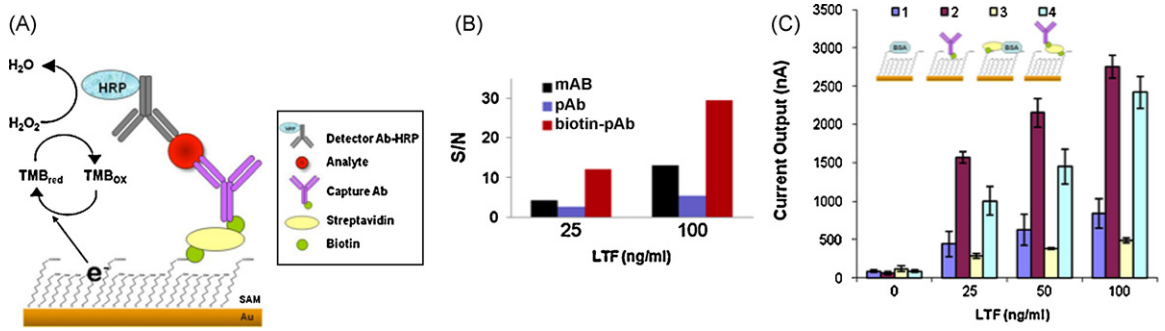

Fig. 1. (A) Schematic of the electrochemical immunosensor assay, with sandwich detection of the analyte by a biotinylated capture antibody and a HRP-conjugated detector antibody. (B) Comparison of the binding affinity of three different LTF capture antibodies directly conjugated to the SAM: a monoclonal Ab (mAb), a polyclonal Ab (pAb), and a biotinylated polyclonal Ab (biotin-pAb). The biotin-pAb has the best signal-to-noise (S/N) ratio for electrochemical detection of LTF. (C) Comparison of capture antibody immobilization techniques through *direct* covalent binding to the SAM (2, red) or *indirect* binding thorough a biotin-streptavidin-biotin linkage (4, light blue). Non-specific signals for both immobilization strategies were measured by substituting capture antibody with BSA (1, dark blue and 3, yellow). Each bar represents the mean value from duplicate experiments.

anti-LTF polyclonal antibody was added and incubated at 37 °C for 1 h. Finally, plastic wells were attached to the sensor chip and 50 μl of substrate solution was delivered to each biosensor using a multi-channel pipettor. Amperometric measurements (current versus time) were immediately and simultaneously taken for all 16 sensors at −200 mV.

2.6. ELISA Protocol

LTF ELISA was performed with different combinations of commercially available monoclonal and polyclonal anti-LTF antibodies as well as a commercially available ELISA kit (Calbiochem, San Diego, CA). Urine samples were diluted to 5-, 20-, and 100-fold in the sample dilution buffer provided in the kit and loaded in duplicate into the ELISA plate along with appropriate concentrations of calibration standard. After incubation for 1 h at 37 °C, the wells were washed five times with approximately 300 μl of wash buffer, followed by the addition of 100 μl of biotinylated anti-LTF solution to each well. The plate was incubated again for 1 h at 37 °C followed by washing (5×) with the wash buffer. Next, 100 μl of streptavidin-HRP solution was added to all wells and incubated for 15 min at 37 °C. After repeating the washing step, substrate (100 μl) was added to each well allowing for reaction with HRP for 5–10 min at 37 °C. Finally, 50 μl of stop solution (1 M H_2SO_4) was added to each well and the optical absorbance was measured.

2.7. Urine samples

The urine collection research protocol was approved by the Stanford University Institutional Review Board and by the Veterans Affairs Palo Alto Health Care System (VAPAHCS) Research and Development Committee. From July 2007 to April 2009, urine samples were collected from patients at the VAPAHCS Spinal Cord Injury (SCI) Unit, who were at high risk for UTI. For each patient sample, one aliquot was brought to our laboratory and one was sent to the clinical laboratory for urinalysis (microscopy and dipstick) and urine culture. Urine microscopy provides information including the degree of pyuria by quantifying the number of white blood cells per high power field (WBC/HPF). Urine dipstick analysis includes pH, specific gravity, leukocyte esterase, and presence of nitrite. Urine culture provides bacterial identification and concentration reported as colony forming units per ml (CFU/ml). Samples were received in our laboratory within 2 h of collection, divided into 1 ml aliquots, and transferred to a −80 °C freezer for storage. For the spiked urine samples, healthy subjects were recruited and known concentrations of purified LTF were added.

2.8. Simultaneous pathogen identification and LTF assay

The electrochemical immunoassay to quantify LTF in clinical urine samples was combined with a molecular pathogen identification assay based on our previously described sandwich hybridization of DNA oligonucleotide probes against bacterial 16S rRNA (Liao et al., 2006, 2007). Sensors 1–8 on the electrochemical biosensor were functionalized with DNA capture probes against all bacteria (UNI), *E. coli* (EC), *Enterococcus* species (EF), *Klebsiella-Enterobacter* group (KE), *P. aeruginosa* (PA), *P. mirabilis* (PM), *Enterobacteriaceae* group (EB), and *Acinetobacter baumannii* (AB) and the anti-LTF capture Ab (sensors 9–16) at 37 °C for 30 min. The probe sequences and the detailed protocol for pathogen identification targeting bacterial 16S rRNA is described elsewhere (Liao et al., 2006). Urine lysate for pathogen detection was deposited on sensors 1–8 and LTF standards and diluted urine samples were deposited on sensors 9–16. Anti-fluorescein HRP targeting the detector probe and HRP labeled anti-LTF detector Ab were then added to sensors 1–8 and 9–16, respectively. The biosensor was incubated at 37 °C for 30 min, followed by addition of HRP substrate solution for simultaneous electrochemical pathogen and LTF detection.

2.9. Statistical analysis

The limit of detection (LOD) was defined as the amount of LTF required for amperometric signals two times the pooled standard deviation over background in $\log_{10}$ units. The LTF concentration determined by the biosensor and the ELISA were compared in the clinical urine specimens. Pearson's correlation coefficient and the 95% confidence interval were calculated. Specimens with LTF concentrations above 10 μg/ml were excluded from this calculation because their signals from 100-fold dilution were above the upper linear detection range of 100 ng/ml and thus the actual concentrations were not known. To assess validity, the LTF concentrations determined from the biosensor were compared with between groups according to standard urinalysis parameters from the VAPAHCS clinical laboratory, including number of WBC/HPF (0–2, 3–10, 11–50 and greater than 50), and leukocyte esterase activity (N = none, 1, 2, 3), using the Wilcoxon rank-sum test and the Kruskal–Wallis test were performed to compare the LTF concentrations between the groups. The Jonckheere–Terpstra test was also performed to test monotone trends of LTF concentrations with respect to WBC/HPF and leukocyte esterase activity, for example, whether LTF concentrations increased with increasing WBC/HPF. $P \leq 0.05$ was considered statistically significant. All tests were two-sided. SAS version 9.1.3 (SAS Institute Inc., Cary, NC) was used for the analyses.

3. Results and discussion

3.1. Characterization of the biosensor coated with self-assembled monolayer

In our previous report, we used the biosensor array coated with a homogeneous SAM based on MUDA for the detection of bacterial-specific nucleic acid target 16S rRNA (Liao et al., 2006, 2007; Mach et al., 2009). Recent work by others (Wittmann and Alegret, 2005) suggested that a mixed SAM composed of alkanethiols and shorter spacer molecules may improve detection sensitivity by reducing steric hindrance for binding of the molecules that are covalently conjugated to the carboxylic-terminated alkanethiols. We have found empirically that compared to homogeneous MUDA SAM, a mixed SAM composed of MUDA and MHOH spacer molecule at 1:5 molar ratio yielded 2.5-fold improvement in SNR for nucleic acid detection using the biosensor (V. Gau, unpublished data).

To further characterize the difference between homogeneous MUDA and mixed MUDA:MHOH SAM, electrochemical impedance analysis was performed (Supplementary Fig. 1). The SAM layer is considered as a capacitive element and a resistive element in parallel. The resistive element typically has a large value and the impedance is dominated by the buffer conductivity at high frequency. At an intermediate frequency, higher impedance for the MUDA chip indicated a smaller capacitance for the MUDA coating, which has a larger thickness. The electrochemical impedance spectra at low frequency data indicated that the space coating had a low resistance value, which can potentially increase the signal level of the sensor (Anandan et al., 2009). This is supported by the amperometric data, which shows the amperometric readout of the two different SAM conjugated to biotin followed by serial dilution of streptavidin-peroxidase. The MUDA:MHOH chips yielded signals 4-fold higher over 4-logs. Based on the impedance analysis and the amperometric data, SAM based on mixed MUDA–MHOH was selected for LTF immunoassay development.

3.2. Development and optimization of the lactoferrin electrochemical immunoassay

A schematic of the electrochemical biosensor assay for detection of LTF is shown in Fig. 1A. Similar to a sandwich ELISA, the biosensor assay uses a capture Ab immobilized on a solid support. Binding of the analyte provides a recognition site for binding of an HRP-conjugated detector Ab. For the biosensor assay, addition of the substrate solution and a fixed voltage between the working and reference electrodes generates an amperometric signal proportional to the number of HRP-conjugated detector Abs present. To maximize the sensitivity of the biosensor assay we optimized several parameters including the Ab pairs, sensor surface linkage and reagent concentrations.

In sandwich ELISA and the electrochemical biosensor assay, the detection sensitivity depends critically on the performance of the capture and detector Ab pairs. We first evaluated the affinity of the specific capture Ab against LTF. Three different anti-LTF Abs, a monoclonal Ab (mAb), a polyclonal Ab (pAb), and a biotinylated polyclonal Ab from a different source (biotin-pAb), were compared. The capture Abs were directly conjugated to the activated SAM and incubated with purified LTF, followed by a common HRP-conjugated detector Ab. As shown in Fig. 1B, the biotin-pAb demonstrated the highest SNR, which was also confirmed by complementary ELISA experiments (data not shown).

We next evaluated the optimal method to immobilize capture antibody to the sensor surface. Our previous work with nucleic acid detection for pathogen identification used biotinylated capture probes to indirectly bind to the SAM-coated gold sensor surface modified with biotin and streptavidin (Au-SAM-biotin-SA-biotinylated-DNA probe) (Liao et al., 2006). For the LTF assay, we compared two different methods to immobilize the capture antibody: *direct* covalent (Au-SAM-biotinylated-pAb) or *indirect* (Au-SAM-biotin-SA-biotinylated-pAb). Fig. 1C shows there were minimal differences between the direct and indirect method. Since our nucleic acid detection is based on the indirect method of immobilizing the capture DNA probes, the indirect method is chosen for the LTF assay development to facilitate the surface functionalization steps for integration of the two assays. Lastly, we examined optimal capture Ab concentration (0.1–40 μg/ml) and incubation time (1–4 h) on the sensor surface. Capture Ab concentrations of 5 μg/ml with 1 h incubation were found to be optimal (data not shown).

Using the optimized parameters described above, the limit of detection (LOD) for LTF using the electrochemical immunosensor was determined to be 145 pg/ml with a dynamic range of 3-orders of magnitude. The LOD is an improvement over the commercially available ELISA kit for LTF detection (1 ng/ml) and prior reports of LTF detection using ELISA (Arao et al., 1999).

3.3. Lactoferrin detection in spiked urine

After establishing conditions for biosensor detection of LTF in buffer, we sought to detect LTF in urine. First, LTF at 25, 50, and 100 ng/ml was spiked in to urine samples obtained from three healthy individuals. Compared to buffer spiked with LTF, the signals for normal urine spiked with LTF were on average 30% lower across the different LTF concentrations (data not shown). We hypothesize that the lower signals are due to urine matrix effect, as urine contains a complex mixture of proteins and a high concentration of electrolytes (Sviridov and Hortin, 2009). To test our hypothesis, the urine samples were diluted with PBS and we found that a 2-fold dilution of urine increased the recovery rate for 50 ng/ml LTF from 66.4 to 88.6% (data not shown), suggesting the presence of inhibitory urine matrix effect.

3.4. Electrochemical immunosensor assay of lactoferrin in infected urine samples

A key aspect in the development of a biosensor application beyond the realm of proof-of-principle is validation with clinical samples derived from the appropriate patient population. We evaluated LTF detection in clinical urine samples using the electrochemical biosensor. Pyuria, or the presence of WBC's in urine, has long been established as a UTI marker (Antwi et al., 2008; Hiraoka et al., 1995). As one of the proteins secreted by the WBC's, LTF has antimicrobial properties and competes with pathogens for scavenging of iron, which is important for pathogen survival (Farnaud and Evans, 2003; Ward et al., 2005). Pyuria is typically determined by urine microscopy and reported as the number of WBC's per high power field (WBC/HPF), which requires an experienced laboratory technician counting the WBC under the microscope using the 40× objective. Pyuria may also be indirectly determined as part of the urine dipstick to measure the enzymatic activity of another WBC-derived protein, leukocyte esterase (LE). When the dipstick containing the enzymatic substrate of LE is in contact with urine containing WBC, a colorimetric change results (reported as *N*one, 1+, 2+, 3+), which corresponds to the degree of pyuria.

To validate LTF as a pyuria marker, we examined urine samples collected from an outpatient spinal cord injury (SCI) clinic. SCI patients are at significant risk of recurrent UTI due to their neurological impairment of bladder emptying (Linsenmeyer and Oakley, 2003). A total of 111 urine samples from 106 patients were tested. On each sensor array, a urine sample was tested at different dilutions (undiluted, 5-, 20-, and 100-fold dilution). A standard

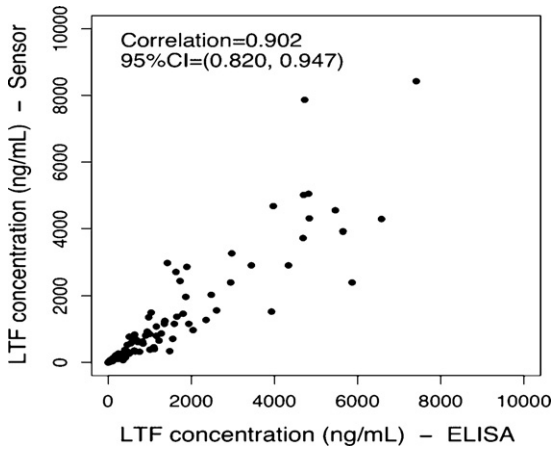

Fig. 2. Correlation of urinary LTF concentration as measured by ELISA (*X*-axis) and the electrochemical immunosensor (*Y*-axis) in 111 clinical urine samples. The correlation coefficient was found to be 0.902. Abbreviation: CI = confidence interval.

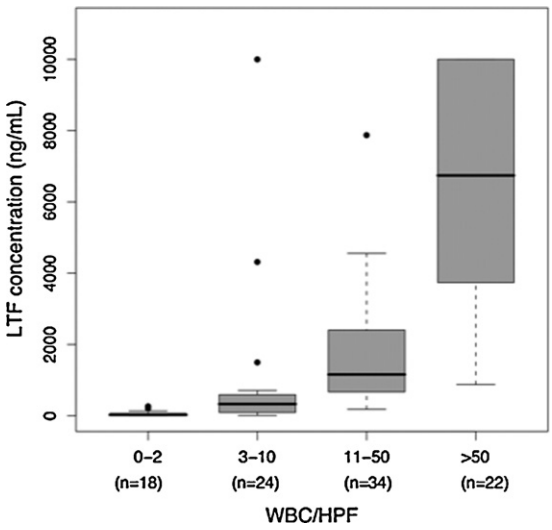

Fig. 3. Comparison of urinary LTF as measured by the electrochemical immunosensor with pyuria (presence of WBC in urine) based on urine microscopy by the clinical laboratory. Box plot of the LTF concentration stratified by urine microscopic measurement of white blood cells per high power field (WBC/HPF).

curve was generated for each urine sample using LTF standards at 0, 3.1, 25, 100 ng/ml to order to calculate the LTF concentration. Each experimental condition was done in duplicate on an individual 16-sensor array (8 conditions × 2 = 16 sensors). A parallel ELISA experiments was performed for each urine sample. Fig. 2 shows a scatter plot of the LTF concentrations as measured by the biosensor assay and ELISA. Among the 111 samples, 12 had LTF concentrations greater than 10 μg/ml based on ELISA, which is beyond the upper limit of the linear detection range of the biosensor and excluded from the analysis. Of the remaining 99 samples, the correlation coefficient between the biosensor assay and ELISA was 0.90 (95% confidence interval, 0.82–0.95). Representative electrochemical immunoassay results are shown in Supplementary Fig. 2.

We examined the relationship between urinary LTF concentrations in the 111 clinical samples, as measured by the electrochemical immunosensor, with the clinical laboratory reported results of urine microscopy for WBC. To facilitate interpretation, the WBC concentration was divided into four groups. Fig. 3 shows a dose–response relationship between LTF level and WBC concentration and was found to be statistically significant

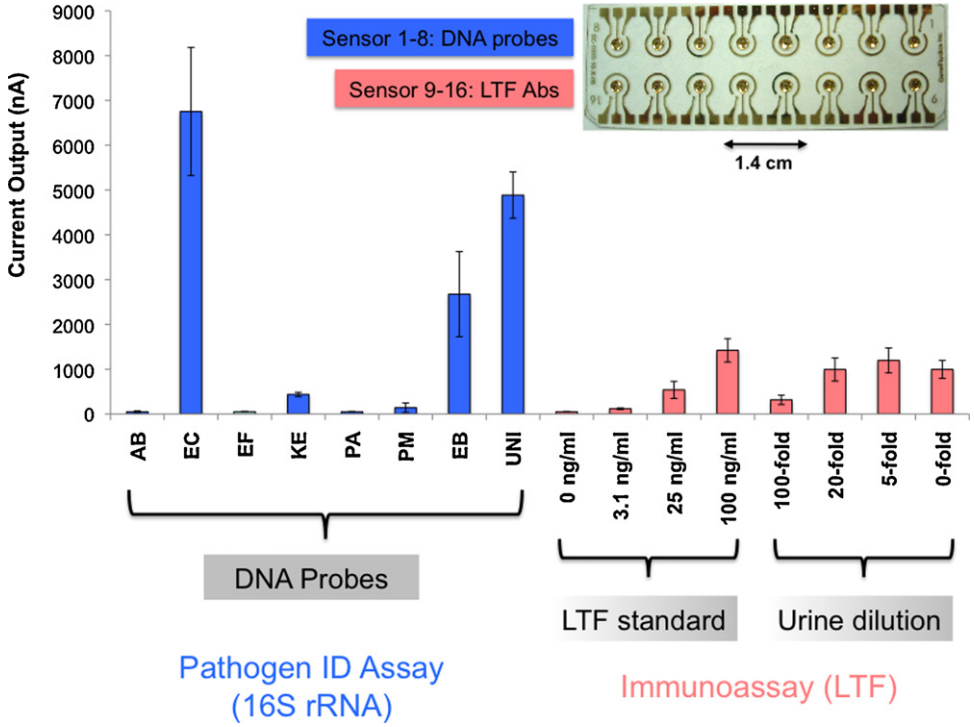

Fig. 4. Simultaneous detection of bacterial 16S rRNA and LTF from an infected urine sample on a single 16-sensor chip. Sensors 1–8 are functionalized with DNA probes against common urinary pathogens: AB, *Acinetobacter baumannii*; EC, *Escherichia coli*; EF, *Enterococcus* species; KE, *Klebsiella-Enterobacter* group; PA, *Pseudomonas aeruginosa*; PM, *Proteus mirabilis*; EB, *Enterobacteriaceae* family; UNI, universal bacterial. Sensors 9–16 are functionalized with LTF capture antibody. HRP is used in both assays for amperometric measurement. *E. coli* was identified based on the amperometric signals of the EC probe and the positive control EB and UNI probes. Urinary LTF level was calculated to be 1208 ng/ml, which corresponded to significant pyuria. The error bars represent duplicate experiments on two sensor chips. The overall coefficient of variation for the sensor assays was 0.13. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

($P < 0.001$). Similar dose–response relationship and statistical significance was found between LTF and LE as measured by dipstick analysis (Supplementary Fig. 3).

A potentially powerful application of the electrochemical detection platform is simultaneous detection of nucleic acid and protein targets using a single array, given the similarity of functionalizing the sensor with biotinylated recognition elements (capture DNA probes and antibodies) on the mixed SAM surface and the common amperometric detection using HRP. We examined the feasibility of immobilizing both capture DNA probes and antibody against bacterial 16S rRNA and host LTF, respectively, on the same sensor array. Fig. 4 shows 1 of 5 integrated pathogen and LTF assays conducted using an urine sample suspected to be infected. The simultaneous assay identified *E. coli* (signals in EC probe and positive control probes EB and UNI) and 1208 ng/ml of LTF in the urine. The entire assay was completed on a single sensor array within 2 h of receiving the urine sample, providing both the pathogen identification based on 16S rRNA as well as the LTF level, a significant improvement over the standard 2-day requirement for the clinical laboratory to perform urine culture. Based on these promising results, we anticipate starting a larger prospective clinical study to evaluate UTI diagnosis and severity assessment using the combined assay.

Measurement of urinary biomarkers such as LTF is currently not part of routine clinical evaluation of UTI. While urine microscopy and dipstick analyses are relatively fast and inexpensive, their disadvantages include imprecision, inter-operator variations, and lack of multiplex capability. To add ELISA to measure urinary biomarkers would result in four different tests for a single urine sample (i.e. microscopy, dipstick, culture, and ELISA). Additional barriers include absence of validated biomarkers and a flexible diagnostic platform in which the biomarkers, once validated, can be easily integrated. Our sensor platform offers an attractive approach to incorporate urinary biomarker detection as part of UTI diagnosis. The multiplex capability to perform simultaneous assays on a single detection platform will facilitate the integration of automated sample preparation with microfluidic technology which we are currently investigating. Furthermore, as additional biomarkers (nucleic acids or protein) are identified and validated (Nanda and Juthani-Mehta, 2009; Zaki Mel, 2008), they can be readily integrated into our sensor platform. Our findings represent an important step toward a comprehensive point-of-care biosensor platform that includes quantitative pathogen identification and measurement of host immune response.

4. Conclusion

In this study, we described a systematic analytical and clinical validation of a SAM-coated electrochemical biosensor platform for detection of UTI biomarker LTF. A limit of detection of 145 pg/ml was achieved with minimal sample preparation steps. Clinical validation of LTF detection was performed in 111 patient-derived urine samples, which correlated with pyuria, an important hallmark of UTI. We further demonstrated in pilot experiments multiplex detection of both bacterial-specific nucleic acids (16S rRNA) and host immune response protein (LTF) on a single array. Our detection platform has the potential to deliver diagnostic information not only more expeditiously but also more informative than the current standard.

Acknowledgments

We thank nursing staff at VAPAHCS spinal cord unit, Christine Du, Mey Yip for assistance with urine sample collection, Mey Chiu, Ph.D. and Govind Kaigala, Ph.D. for critical reading of the manuscript. This work is supported by Department of Veterans Affairs RR&D Merit Review (B4872), NIH/NIAID (1U01AI082457), and NSF ECCS-0901292 awarded to J.C.L.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.07.002.

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