Quantitative urinary tract infection diagnosis of leukocyte esterase with a microfluidic paper-based device

Wei-Ting Tseng, a Hsin-Yi Tseng, a Yin-Yu Chou, a Yin-Chen Wang, a Tz-Ning Tseng, a Li-Ing Ho, b,c Sheng-Wei Pan b *,c and Mei-Lin Ho d *,a

Leukocyte esterase (LE) is a useful marker that can be used in establishing a diagnosis of urinary tract infections (UTIs). The development of a UTI diagnostic method with quantitative determinations of biomarkers across all age groups is becoming more important. In this report, microfluidic resistance sensors based on silver ink (Ag ink) and silver ink mixed with ZnO nanoparticles (Ag-ZnO ink) were synthesized and coated on cellulose paper, namely LE-Ag-μPADs and LE-Ag-ZnO-μPADs, respectively, for the sensitive detection of LE. The microfluidic design increases the precision of data and further allows for quantitative determination and early detection of LE in human urine. The quantification of LE relies on the change in the resistance readout coating with Ag ink as well as Ag-ZnO ink in the detection zone. A mixture of 3-[(N-tosyl-L-alanyl-oxy)-5-phenylpyrrole (PE) and 1-diazo-2-naphthol-4-sulfonic acid (DAS) was deposited in the sample zone to selectively recognize LE, and the resulting nonconductive products, i.e., azo compounds, further reacted with the Ag ink and Ag-ZnO ink to increase resistance. The quantitative detectable LE concentrations between 2 to 32 (×5.2 U mL⁻¹), i.e., ±12 to 108 μg L⁻¹, cover the commercial dipstick range of trace, +1 and +2. The minimum detectable concentration of LE in urine was 1 (×5.2 U mL⁻¹). The lower concentrations of LE detectable by LE-Ag-μPADs (1–8 × 5.2 U mL⁻¹) are below the value achieved with the ELISA LE kit. Urine samples from inpatients with indwelling urinary catheters were used, and the LE levels measured by the present device were highly correlated with those determined by a commercial urine analyser.

Introduction

Urinary tract infections (UTIs) are among the most common types of infection in the body. Leukocyte esterase (LE) is a leukocyte protein, and it is tested for in urine as a screening test for white blood cells associated with UTIs. Commercial dipstick urinalysis (i.e., LE test) is frequently used by physicians to screen for the presence of pyuria and suspected uncomplicated UTIs. ¹,² The LE assay in dipstick form is based on the enzymatic cleavage of an indoxyl ester on the strip, and the released indoxyl reacts with a diazoniunm salt to form an azo dye that changes the colour to purple.³

The literature on the development of quantitative LE methods is scarce. Recently, an automated urine analyser based on complementary metal oxide semiconductor technology for the quantitative analysis of urinary test strips was reported.⁴ On the other hand, among the methods for UTI diagnosis, electrochemical sensors provide high sensitivity, simple operation, low cost, and fast response and thus have attracted considerable attention.⁵ The first quantitative measure of LE by electrochemical assay was proposed by W. Gorski’s group.⁶ The assay is based on the LE-induced discharge of synthesized substrate, which oxidizes at an electrode. A recent strategy to develop quantitative LE assay comprises the use of chronocoulometry on a glucose strip and a hydroquinone-releasing substrate (HQS), in which LE-triggered hydrolysis of HQS is detected from the released HQ and strip as a signal transduction.⁷ The other substrates, such as methyl pyruvate and glucosyl esters, in conjugation with alcohol oxidase and a nitrogen-doped carbon nanotube electrode, allow for the detection of LE.⁸,⁹ Previously, we reported a paper-based analytical device by chemiresistive method that can be used as a quantitative test of LE.¹⁰

The use of paper substrates in point-of-care (POC) testing has become attractive in biosensor research.¹¹ Diversified...
analytical techniques for quantitative analysis as signal readouts used in paper-based sensors include the following: colorimetry, luminescence, conductivity, and electrochemistry.\textsuperscript{11-13} When microfluidic channels are built on paper, the liquid flow can be guided in a controlled manner, which enables the devices to act as analytical platforms for assays, either multi-analyte or semi-quantitative/quantitative analysis.\textsuperscript{14} Integration of sensing materials with paper-based microfluidic devices (µPADs) that undergo electrochemical reaction in the presence of an analyte results in resistance and conductance changes,\textsuperscript{15} presenting ideal candidates for health care, forensic analysis, medical diagnostics and environmental monitoring applications. For instance, the Whitesides and Henry groups have employed microfluidic paper-based electrochemical devices for quantifying the glucose in aqueous solutions.\textsuperscript{16,17} Suresh et al. fabricated µPADs for the ultra-low detection of urea through urease catalysis.\textsuperscript{18} Recently, Yukird et al. reported on a 3D µPADs for dual detection of bisphenol A via electrochemical and laser desorption ionization mass spectrometric detection.\textsuperscript{19} Fukana et al. proposed a method for quantifying sulfite preservative by a contactless conductivity sensor for microfluidic paper-based analytical device.\textsuperscript{20} Pungjunun et al. reported a laser engraved microapillary pump paper-based microfluidic device for colorimetric and electrochemical detection of salivary thiocyanate.\textsuperscript{21}

Flexible electronics using conductive ink provide high electrical conductivity and large surface areas, so they have attracted tremendous research interest. Andersson et al. injected silver nanoparticle ink to develop a humidity sensor on paper.\textsuperscript{22} Similarly, Barmpakos et al. printed silver nanoparticle ink and PEDOT:PSS onto photo glossy paper to measure relative humidity, temperature, and compressive and tensile bending.\textsuperscript{23} Hwe et al. proposed a graphene and silver nanoparticle-based strain sensor on a polyvinyl alcohol substrate.\textsuperscript{24} M. M. Ali developed a silver nanowire/silver flake strain sensor on thermoplastic polyurethane substrate.\textsuperscript{25} Although several applications of silver ink have been proposed, the use of silver ink to fabricate biosensors for POC testing is uncommon. On the other hand, to increase the detection sensitivity, the detection devices and materials need to be modified. Previously, Chung et al. found that sintered Ag/Cu film has higher electrical conductivity and oxidation stability than those of Cu nanoparticles.\textsuperscript{26} Huang et al. added Ag nanoparticles into ZnO and noted increases in electrical conductivity of up to 1000 times.\textsuperscript{27} Ramesan et al. incorporated Ag-ZnO in a conducting polymer to increase the alternating current (AC) conductivity and dielectric properties.\textsuperscript{28} Shrivas et al. designed a piezoelectric touchpad by depositing ZnO on the conductive track of Ag nanoparticles.\textsuperscript{29} ZnO nanoparticles have a band gap at 3.37 eV, high UV absorption ability, a large surface area, high biocompatibility, and good electrocatalytic activity, so they are suitable for improving sensor performance.\textsuperscript{18,29}

Ag-based ink exhibits admirable flexible and electrical properties. Compared to bare Ag ink, Ag-ZnO-based ink increased conductivity and electrocatalytic properties. Herein, in this study, silver ink with or without ZnO decoration was selected as an active layer on µPADs for the detection of LE. The results show that pristine silver ink exhibits higher sensitivity than that of silver ink with ZnO (vide infra). The device based on silver ink was used to detect LE in patients’ urine.

### Results and discussion

#### Sensor fabrication of LE-µPADs

To develop better sensitivity and a quantitative method to recognize LE, the battery configuration with a microfluidic channel was used for the LE-sensing design (LE-µPADs, Fig. 1(a)) to investigate the effect of structural change and the composition of the active layer, \textit{i.e.}, silver ink (LE-Ag-µPADs, Fig. 1(b)) and silver ink with ZnO nanoparticles (LE-Ag-ZnO-µPADs, Fig. 1(c)), on the electrochemical response of the sensor. The pattern design of the µPADs and the resistance change for the sensors deposited with different transduction components when exposed to different LE concentrations are shown in Fig. 1. The schematic fabrication of the µPADs is shown in Fig. 1(d). The device LE-µPADs is composed of three zones. In the sample zone, Fig. 1(a), the mixture of PE and DAS is deposited, and a microfluidic channel connects the sample and the detection zone. Then Ag ink and Ag-ZnO ink are deposited onto the detection zone, respectively (Fig. 1(b) and (c)).

#### Sensing mechanism of the LE-µPADs

The surface morphologies of the LE-Ag-µPADs and LE-Ag-ZnO-µPADs, determined by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDX), are shown in Fig. 2. The SEM of the pure paper substrate was taken for comparison.
From the SEM (Fig. 2(a)) images of Ag ink, it can be seen that the nanosphere particles and the nanowires formed neck-like junctions between neighboring nanoparticles under pulsed light sintering. Compared to the synthesized Ag ink without sintering, it clearly shows that the organic binder around the AgNPs was reduced by the sintering process. The EDX spectrum of the sintered Ag ink shows clear peaks indicating carbon, oxygen and silver, confirming that the Ag film comprised silver nanoparticles. The SEM image and EDX analysis of the Ag-ZnO ink are shown in Fig. 2(b). Under sintering of pulsed Xenon light, the Ag-ZnO ink had a prominent grainy and stick structure (Fig. 2(b)). In addition to nanospheres and nanowires, more short rod-shaped nanoparticles appeared, and the density of nanoparticles was higher than that of Ag ink. At the same time, the relative content of zinc appeared and that of oxygen increased, which demonstrated that ZnO with AgNPs was deposited on the paper.

When the concentrations of LE in the sample zone of LE-Ag-\(\mu\)PADs were increased, as shown in Fig. 2(c), (d), and (e) and compared with 2(a), the amount of nanospheres increased and the width distribution of the nanowires in Fig. 2(e) increased from 36–55 nm to 64–73 nm. The relative content of silver decreased, but those of oxygen and sulfur increased, with more LE addition. These alterations, i.e., the sensing mechanism, appeared because of product azo compound formed and covered the Ag ink. This insulating layer on the Ag ink caused a change in resistivity. The UV spectrum was further used to analyse the azo compound formation on the response region. When the amount of LE in the sample zone was increased, the spectra showed that the colour purple appeared on the detection zone, and the absorption band around 580–700 nm increased, which indicated the formation of the azo compound (Fig. 3).

The FTIR spectrum of LE-\(\mu\)PADs upon addition of LE is presented in Fig. S2 in the ESI†. The absorption peaks were situated at 3433, 2925, 1634 and 1410 cm\(^{-1}\), which respectively corresponded to the \(\text{–OH}\) stretching vibration, the stretching vibration of N–H groups, the R–N\(\equiv\)N–R (azo), and the S\(=\)O stretching vibration of sulfonamide groups. As displayed in the FTIR spectrum of Ag-\(\mu\)PADs without LE added, i.e., Ag ink, the absorption peaks were respectively situated at 3320 and 1613 cm\(^{-1}\), indicating the \(\text{–OH}\) stretching vibration and the C\(=\)O stretching vibration. As compared to LE-\(\mu\)PADs, those

![Fig. 2](image1)

**Fig. 2** SEM images and EDX analysis of (a) Ag ink film, (b) Ag-ZnO ink film; (c, d and e) three different concentrations of LE (6, 20, 38 \(\times\) 5.2 U mL\(^{-1}\)) were added to LE-Ag-\(\mu\)PADs films.

![Fig. 3](image2)

**Fig. 3** Absorption spectrum of LE-Ag-\(\mu\)PADs and that of the reaction product, azo compound; the absorbance increased with different concentrations of LE (6, 20, 38 \(\times\) 5.2 U mL\(^{-1}\)).
were attributed to the –C=O and –OH of PVP capped Ag film. With an increased concentration of LE on the LE-Ag-µPADs, in comparison to Ag ink, the peaks at 2916–2927 cm\(^{-1}\), 1626–1639 cm\(^{-1}\) and 1397–1407 cm\(^{-1}\) were enhanced. They were the absorption peaks of N–H, R–N=N–R (azo) and S=O stretching of the azo compound.

On the other hand, the smaller change at the same range in absorbance of the LE-Ag-ZnO-µPADs implied less azo compound formation compared to that on the Ag ink when the LE concentration increased (Fig. S3†). This might have been due to the photodegradation of azo under UV irradiation in the presence of ZnO.\(^{30}\) Upon addition of different concentrations of LE to LE-Ag-µPADs and LE-Ag-ZnO-µPADs, the resistance change of the LE-Ag-ZnO-µPADs was not as large as that of the LE-Ag-µPADs (Fig. 4). Simultaneously, the SEM-EDX data of the LE-Ag-ZnO-µPADs show that the amount of sulfur gradually increased (Fig. S4†) upon addition of LE, which also indicated the formation of azo compounds on the Ag-ZnO layer. From the above, it is clear that the LE-Ag-µPADs and LE-Ag-ZnO-µPADs have the same sensing mechanism for LE, and also that the resistive change of the LE-Ag-µPADs has a better response to LE, so this device was chosen for further experiments.

Characterization and optimization of LE-Ag-µPADs

First, different types of papers, including Advantec quantitative filter paper no. 5C, Whatman cellulose nitrate membrane filters, and Whatman qualitative filter paper no. 1 were used to investigate the signal changes upon addition of LE into the sample zone. Amongst all the papers, the Advantec quantitative filter paper no. 5C in LE-Ag-µPADs resulted in a better signal change. Due to the rather small pore size and slow flow rate of the selected paper, the elution solvents (buffer solution at pH 7.0, deionized water and 95% EtOH) for LE were applied subsequently to improve the elution of products from the sample zone to the detection zone. In Fig. S5(a),† the relative resistance changes in the detection zone are plotted against the elution solvents. Though the relative resistance change with the buffer solution was the highest, 95% EtOH was selected as the elution solvent due to the large standard deviation with buffer solution and water. We then investigated the volume effect of EtOH on the signal change of LE-Ag-µPADs. Elution of a large volume of solvent increased the signal change, and elution in 10 µL resulted in a small standard deviation of the signal change (Fig. S5(b)). Next, the effects of the LE volume (8–20 µL) and buffer pH (5.3–8.0) in response to 8 × 5.2 U mL\(^{-1}\) LE were examined, as shown in Fig. S5(c) and (d).† With a higher volume of LE, e.g., higher moles of LE, an increase in resistance change was observed. However, due to the high standard deviation of the results obtained from the 12–20 µL of 8 × 5.2 U mL\(^{-1}\) LE and pH 7.0 buffer solution, the optimum volume of LE and pH value of buffer solution were 10 µL and 7.0, respectively. As shown in Fig. S5(e),† the ΔR% was the highest at a buffer concentration of 100 mM. The optimal distance between the sample zone and the detection zone with various distances was characterized to determine the signal change and the precision of the data (Fig. S5(f)).† Fig. S5(f)† shows the relative resistance change for the respective microfluidic distance. As shown in Fig. S5(f),† the signal change was proportional to the distance at the very beginning of the increased channel length. A better signal was observed when the length of the microfluidic channel was increased, and the 0.25 cm distance yielded the best precision of the data.

Analytical performance of the LE-Ag-µPADs

Fig. S6† represents the relationship between the obtained resistance change and LE concentration under the optimum conditions. As expected, the resistance change was enhanced at higher LE concentrations. There were two linear segments in the ranges 2 to 9.3 × 5.2 U mL\(^{-1}\), i.e. ≈ 12 to 55 µg L\(^{-1}\), and 9.4 to 32 × 5.2 U mL\(^{-1}\), i.e. ≈55 to 108 µg L\(^{-1}\), respectively. The calibration equations obtained were ΔR% = 0.075[LE] – 0.062 and ΔR% = 0.015[LE] + 0.498, respectively (R\(^2\) = 0.9933 and 0.9871). The formation of the non-conductive compound, i.e., the azo compound, increased, which led to the increase in the resistivity in the first linear concentration region. When the LE further increased, accompanied by the consumption of the remaining PE/DAS in the sampling inlet region, the activated surface area for the silver ink decreased due to the adsorption of related products during the accumulation step. As a result, the sensitivity decreased at higher LE concentrations. The detection limit calculated from the first region was 0.8 × 5.2 U mL\(^{-1}\), i.e. ≈3 µg L\(^{-1}\), which was calculated from 3σ/S, where σ is the SD of three independent experiments and S is the slope of the calibration curve.

Fig. S6† also shows the correlation between the content of LE determined by LE-Ag-µPADs and changes in colour intensity in the commercial LE dipstick. The sensing range correlated the LE concentration with the current commercial dipstick from trace, +1 and +2 regions. The “trace” in urine ranged from 1 to 4 × 5.2 U mL\(^{-1}\), “+1” from 4.1 to 15 × 5.2 U mL\(^{-1}\), and “+2” from 15.1 to 32 × 5.2 U mL\(^{-1}\), respectively.
The analytical performance of our assay is compared with those of other LE detection methods in Table 1. Although other electrochemical methods have a wider detection range of LE and can expand to the +3 zone, our device has a lower detection limit (LOD), which can allow early quantitative detection of LE or UTIs. Furthermore, our method has better accuracy and precision in determining LE, and it especially can be applied in the diagnosis of those at greatest risk for UTIs, namely, women and infants. Furthermore, the proposed device provides an advantage in detecting and quantifying the content of LE, and the obtained linear range had a wider range than that of our previous assay.10 This can be rationalized by the microfluidic channel being constructed on a paper-based assay device, which can lower the matrix effect on the analyte.

**Interference study**

The selectivity of the LE-AgμPADs was validated by using potential interferents (ascorbic acid, aspartic acid, glucose, glutathione acid, glycine, KCl, methionine, tryptophan, urea, uric acid and valine) in urine. In the LE-assay, 10 mM of each interfering species, which is largely higher than the average concentrations in healthy individuals, was evaluated on the LE-AgμPADs against 8 (×5.2 U mL−1) LE. Fig. S7† illustrates the relative resistance changes recorded in the presence of interfering ions at concentrations higher than normal in healthy individuals. With albumin as a model protein, it was observed that albumin has no obvious effect on the relative resistance change. This further reinforces the microfluidic design and helps to exclude the potential interferences from large biomolecules. The results obtained from the interference study revealed that the LE-AgμPADs sensor had about 0.03 ± 0.001 relative resistance response to the urine from the healthy individuals and retained 0.98 ± 0.02 relative resistance change upon addition to 8 (×5.2 U mL−1) LE into the human urine. Also, the relative resistance change increased to 0.07 ± 0.02 upon 1 (×5.2 U mL−1) LE addition; hence the minimum detectable concentration of LE in urine was 1 (×5.2 U mL−1), i.e. ≈3.75 μg mL−1. When the LE-AgμPADs were exposed to excessive amounts of individual interfering species, the obtained response to LE was still larger in comparison with the interfering ones. Recently, Ko et al. reported that the presence of ascorbic acid can cause significant interference with some urine test strips that use oxidation reaction.32 In the present assay, the sensing mechanism occurs via azo coupling reaction, and the products further react with the Ag ink. Therefore, ascorbic acid did not afford any significant response on the LE-AgμPADs. From the relative resistivity changes in Fig. S7† good selectivity of the LE-AgμPADs toward LE was observed.

**Stability**

As shown in Fig. S8,† the relative resistance change was maintained at about 97.1% of its initial response after four months. This result indicates that the LE-AgμPADs assay has good stability.

**Accuracy and precision of the LE-AgμPADs**

The accuracy of the LE-AgμPADs assay was validated at five concentration levels (0, 4, 8, 10, 16 and 24 × 5.2 U mL−1) in the urine matrix with three replicates. The results presented in Table 2 show that the recoveries ranged from 97–103%, with the coefficient of variation ranging (CV) from 0.5–4.3%. These results indicate the levels of accuracy and precision of the proposed method for quantitative LE analysis.

The reliability of the proposed method using the LE-AgμPADs devices was further confirmed by comparing the results with those from the ELISA method. The concentrations of LE (10, 16 and 24 × 5.2 U mL−1) calculated by our proposed method and ELISA did not statistically differ (paired t test) at a 95% confidence level.

The spiked concentration, e.g. 1–8 (×5.2 U mL−1), cannot be determined with an ELISA kit, but it can be obtained via LE-AgμPADs assay. This also implies that the present LE-AgμPADs method can be used to detect lower concentrations of LE in urine and applied to the early detection of UTIs.

<table>
<thead>
<tr>
<th>Technology</th>
<th>Method</th>
<th>LOD (μg L−1)</th>
<th>Detection range (μg L−1)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemical</td>
<td>Amperometric assay</td>
<td>≈5</td>
<td>≈5–220†</td>
<td>31</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>ICECEAa amperograms</td>
<td>22</td>
<td>22–300</td>
<td>8</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>ICECEAb</td>
<td>9</td>
<td>9–690</td>
<td>6</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Chronocoulometric</td>
<td>25</td>
<td>25–800</td>
<td>9</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Chemiresistive</td>
<td>≈17†</td>
<td>≈18–34</td>
<td></td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Chemiresistive</td>
<td>≈3 (std soln.)a ≈3.75 (urine)b</td>
<td>≈12–55; 35–108c</td>
<td>Our previous work, ref. 10</td>
</tr>
</tbody>
</table>

† The detection range was converted by taking to ref. 6. Std soln.: LE standard solution. a ICECEA was the internally calibrated electrochemical continuous enzyme assay. b The minimum detectable concentration of LE in urine (vide infra).

Table 1 An overview on recently reported electrochemical methods for detection of LE

<table>
<thead>
<tr>
<th>Biofluids</th>
<th>Spiked LE (×5.2 U mL−1)</th>
<th>LE-AgμPADs LE found (×5.2 U mL−1)</th>
<th>ELISA (×5.2 U mL−1)</th>
<th>Recovery (%)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>0</td>
<td>&lt;1†</td>
<td>N.A.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.9 ± 0.2</td>
<td>N.A.</td>
<td>99</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.2 ± 0.1</td>
<td>N.A.</td>
<td>103</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.7 ± 0.1</td>
<td>9.4 ± 4.5</td>
<td>97</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16 ± 0.6</td>
<td>15 ± 1.9</td>
<td>103</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>24 ± 0.6</td>
<td>23 ± 1.2</td>
<td>100</td>
<td>2.6</td>
</tr>
</tbody>
</table>

a N = 3, b C.V.: Coefficient of variation. c The minimum detectable concentration of LE in urine.
Clinical urine sample analysis

Ten urine samples from 10 unique patients with indwelling urinary catheters (IUCs) at the respiratory care centre of Taipei Veterans General Hospital (TVGH) were obtained and tested. LE levels in the urine samples were determined by LE-Ag-μPADs and commercial urine analyser (Table 3). Three of 10 urine samples tested positive for LE (1+ and trace) by urine analyser. The average level of LE measured by LE-Ag-μPADs was higher in the positive samples than in the negative ones (2.9 ± 2.1 versus 1.0 ± 0.02 (×5.2 U mL⁻¹), p = 0.008 by Mann–Whitney U test). For correlation analysis between levels measured by urine analyser and by LE-Ag-μPADs, the Spearman’s rho was 0.899 with a p value of <0.001. These findings imply that LE-Ag-μPADs assay can be a useful method for detecting LE in clinical urine samples.

Conclusions

The early detection and quantitative diagnosis of LE is of great importance, for the level of LE is related to the presence of white blood cells and other abnormalities associated with infection. The literature on the development of quantitative LE methods is scarce. In this work, quantitative detection of LE can be achieved by using the LE-Ag-μPADs chemiresistor with conductive Ag ink. Another conductive layer of Ag ink and ZnO nanoparticles was also compared. In clinical urine samples, the association between LE measured by commercial urine analyser and by the presented device was confirmed. Our results demonstrate the device’s utility in the quantitative detection of LE in the diagnosis of UTIs, and Ag ink-based chemiresistive sensors might open a new avenue for the early detection of LE associated with UTIs.

Experimental section

Materials

Silver nitrate, polyvinylpyrrolidone (PVP), ethanol (EtOH), sodium dihydrogen phosphate, disodium hydrogen phosphate, D-glucose, ascorbic acid, urea, uric acid, L-tryptophan, L-valine, L-methionine, glycine, L-aspartic acid free acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, D-glucose, ascorbic acid, urea, uric acid, L-tryptophan, L-valine, L-methionine, glycine, L-aspartic acid free acid, sodium chloride, glutathione acid and albumins were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylene glycol (EG) and sodium chloride were acquired from J. T. Baker (Phillipsburg, New Jersey). 1-Diazo-2-naphthol-4-sulfonic acid (DAS) was obtained from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). Acetonitrile was purchased from Acros Organic (Geel, Belgium). 3-(N-Tosyl-L-alaninyloxy)-5-phenylpyrrole (PE) was acquired from Angene (London, UK). Leukocyte esterase (LE) and leukocyte esterase ELISA Kit were obtained from MyBioSource (California, San Diego, USA). Zinc acetate dihydrate, sodium hydroxide and absolute ethanol from Sigma-Aldrich were used for the preparation of ZnO nanoparticles (ZnO NPs). All chemicals were used as received without further purification.

Design of LE sensor

Schematics of the LE sensors are shown in Fig. 1. A total of two different series of experiments were conducted in order to understand the sensor behaviour. In series one, namely LE-Ag-μPADs, the LE-Ag-μPADs contains two separated zones, i.e., a sample zone and a detection zone, and is coated with an active layer of silver ink (Fig. 1(b)). A microfluidic channel connects these two regions. The influence of the microfluidic channel on the LE sensing properties is evaluated.

In series two, ZnO NPs were added to the silver ink as the active layer of LE-μPADs, namely LE-ZnO-μPADs (Fig. 1(c)), to probe LE. The sensing properties of these two devices for LE are compared.

Preparation of silver ink and ZnO nanoparticles

The silver ink was prepared according to previously published articles. Briefly, silver nanoparticles were synthesized first. The mixture of silver nanospheres and nanowires was dispersed in ethanol solution. ZnO NPs are synthesized by sol–gel method. First, 439 mg zinc acetate was dissolved in 10 mL ethanol. Then 10 mL ethylene glycol was added and 252 mg oxalic acid was dissolved in 10 mL ethanol, respectively. Both solutions were stirred for 60 min at 45 °C. To mix these two solutions, 10 mL oxalic acid was added drop-wise during vigorous stirring, which continued for 30 min. Then the gel was dried at 80 °C overnight and calcined at 500 °C for 2 h. To further prepare ZnO solution, ZnO was dispersed in ethanol with ultrasonication for 10 min. Ag-ZnO ink film was dried at 75 °C for 1 h and provided the LE detection.

Instrumental

A wax printer (XEROX ColorQube 8880, Norwalk, USA) was used to print a hydrophobic barrier on the paper substrate. The size and shape of ZnO were determined by TEM (JEOL JEM-2100F). Infrared spectroscopy (PerkinElmer Spectrum 100, Waltham, UK) was used to determine various functional groups present in the active layer. For sheet resistivity in the detection zone, four-point probe measurements were used to calculate the resistivity of the active layer. A JSM-7600 field emission SEM/EDX was used for active layer surface characterization (JEOL). The optical properties of the active layer were
determined by UV-vis spectroscopy (Hitachi U-4100). Quantitative two-site sandwich ELISA assay, optical absorbance at 450 nm was measured using a 96-well plate reader (BioTek Epoch2). The human urine was measured with the CLINITEST Status+ Analyser™ (Siemens Healthcare Diagnostics, Erlangen, Germany) and manual read-outs of urine strips (Multistix PRO, Siemens Healthcare Diagnostics).

Series one—fabrication of LE-Ag-μPADs

LE detection patterns were generated using Microsoft PowerPoint and then printed on filter paper using a wax printer. The patterned paper was subsequently heated on hot plate at 60 °C for 1 min and then subjected to pulse light annealing. An intense pulse light with a xenon flash lamp annealed the silver ink and showed a resistance of about 16 Ω cm⁻². Each pulse light’s duration time was 250 ms and 4 pulses were used. As sample pretreatment reagent, a mixture of PE and DAS (10 μL) was deposited on the sample zone and dried at room temperature, after which it was ready to use (LE-Ag-μPADs). A PE : DAS (v/v = 2 : 1) mixture was prepared using 10 mg mL⁻¹ PE in acetonitrile and 5 mg mL⁻¹ DAS in 100 mM PBS buffer (pH 6.9). A series of experiments were applied to examine and to optimize the LE sensing properties, including the type of paper substrate (Advantec quantitative filter paper no. 5C, Whatman cellulose nitrate membrane filters, Whatman qualitative filter paper no. 1), elution solvent, volume of LE, and distance of the microfluid channel.

Series two—fabrication of LE-Ag-ZnO-μPADs

Pre-weighed ZnO nanoparticles (0.1303 mg) were added to the Ag ink (0.2 mL) to achieve a concentration of 8 mM. The nanoparticles were further ultrasonicated for 20 minutes to stabilize them. After that, 6 μL of the dispersed ZnO was mixed with Ag ink on paper to construct Ag-ZnO ink. This process was repeated several times and intense pulse light with a xenon flash lamp was used to obtain better initial electric conductivity on the paper substrate, which was prepared for LE-Ag-ZnO-μPADs.

Characterization

The surface morphology, microstructural evolution and elemental composition of the Ag ink and those of the Ag-ZnO ink were observed with field-emission scanning electron microscopy (FE-SEM) coupled with energy dispersive X-ray spectroscopy (EDX, JEOL JSM-7600F) and stereo microscopy. The characteristic absorption of both inks and changes upon addition of analyte were analysed by UV/Vis absorbance spectrum and Infrared spectroscopy. Integrating sphere which is used for measurement of LE-Ag-μPADs and LE-Ag-ZnO-μPADs upon addition of different LE. For integrating sphere, barium sulfate is used as the internal coating material, and aluminum oxide is used for the standard reflection plates. The characteristic absorption of both inks and changes upon addition of analyte were analysed by UV/Vis absorbance spectrum and Infrared spectroscopy. The infrared spectrum was measured with a PerkinElmer 2000 FTIR spectrometer.

LE assay

Resistivity measurement in the detection zone was used to investigate the electrical conductivity change in the active layer. After fabrication of LE-Ag-μPADs or LE-Ag-ZnO-μPADs, the resistance of the active layer was measured first (initial resistance, R₀). 1–40 μL (×5.2 U mL⁻¹) standard solutions of LE are prepared by diluting 1–40 μL LE solution to a 1 mL volumetric flask and dilute to the mark with pH 7.0 PBS. Following that, the standard solution or sample solutions of LE were introduced in the sample zone. In the presence of LE, the PE and DAS successively reacted to the formation of an azo compound. After 5 min, the 10 μL fluent solvent washed the channel and drove the resulting compounds into the detection zone to react with the active layer. After 15 min waiting to dry the strip, the resistivity was stable and then measured. The resistance in the detection zone was then measured again (R₁), and the resistivity change (ΔR%) was calculated using eqn (1).

\[
\Delta R\% = \frac{R_1 - R_0}{R_0} \times 100\%
\]  

Interference effect

The relative resistance change of uric acid, ascorbic acid, aspartic acid, glutathione acid, urea, KCl, methionine, tryptophan, valine, glycine, glucose and albumins were measured using the LE-Ag-μPADs to detect the influence of interfering substances on LE detection. 60 mg g⁻¹ albumin was prepared in PBS. All interfering substances and a mixture of interfering species were added in concentrations of 10 mM in PBS buffer. The real sample of human urine in the interference study was from a healthy male (age 21).

ELISA protocol

The LE content was measured simultaneously with two-site sandwich ELISA assay and our proposed method. LE solution was spiked into human urine collected from a healthy male (age 21). Human urine in both tests required no dilution. ELISA was performed with a commercially available ELISA kit (MyBioSource, San Diego, CA, USA). Wells of a 96 well assay plate were pre-coated with human LE antibody. 50 μL of appropriate concentrations of standard (31.25, 62.5, 125, 250 and 500 pg mL⁻¹) and sample (0, 4, 8, 10, 16, and 24 × 5.2 U mL⁻¹) were pipetted into the wells, respectively, followed by incubation for 1 hour at 37 °C. Unbound substances were washed 5 times with wash buffer (350 μL) and a 10 μL biotin-conjugated antibody was added to the each well, followed by incubation at room temperature for 1 hour. After washing, 50 μL of Streptavidin conjugated Horseradish Peroxidase was added to each well and the plates were incubated for 1 hour at 37 °C. After washing five times to remove any unbound avidin-enzyme reagent, a 50 μL substrate solution was added to each well, followed by incubation at 37 °C for 10 minutes, and the absorption was measured by ELISA reader.

Urine sample collection

For validation and application to real urine samples, 10 mL urine was collected from each enrolled patient at the
respiratory care center in Taipei Veterans General Hospital (TVGH) in Taiwan. Adult patients with a newly inserted indwelling urinary catheter were consecutively screened and selected for the study from September 2018 to August 2020. Since some enrolled patients had UTI and others did not, UTI status was determined by routine clinical parameters and urine samples were tested by LE-AgµPADs as well as urine analyser. Urine analyser detects leukocyte esterase, which catalyses the hydrolysis of the derivatized pyrrole amino acid ester to release 3-hydroxy-5-phenyl pyrrole. The pyrrole then reacts with a diazonium salt to produce a purple chromophore, and read with the CLINITEK Status®+ Analyser. The study protocol was approved by the institutional review board (IRB) of TVGH, and written informed consent was obtained from each participant (IRB No: 2018–05–006CC). All experiments were performed in compliance with TVGH regulations for ethics and care.

Statistics
Data are presented as mean ± SD. The Mann–Whitney U test was used to compare the groups of non-normal distribution. The correlation between results measured by LE-AgµPADs and urine analyser was tested by Spearman’s correlation analysis, and Spearman’s rho was calculated. LE data from the urine analyser were defined as 0 if the urine tested negative for LE, 0.5 if trace for LE, 1 if 1+ for LE. LE data from the LE-AgµPADs (×5.2 U mL⁻¹) was recorded as 1 if the value was less than 1 in the analyses. SPSS v18 (SPSS Inc., Chicago, IL, USA) was used for all analyses. A p value of <0.050 was considered significant.

Conflicts of interest
There are no conflicts to declare.

Acknowledgements
This work was supported by the Ministry of Science and Technology, Taiwan. The authors are grateful to Ms. S.-J. Ji of the Ministry of Science and Technology (National Taiwan University) for the assistance in the SEM/EDX experiments.

References


