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ARTICLE

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Spectroelectrochemical Sensing of Pyrene Metabolites 1-Hydroxypyrene and 1-Hydroxypyrene-glucuronide

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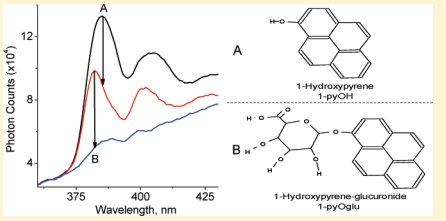
ABSTRACT: Spectroelectrochemical sensing in an optically transparent thin layer electrode (OTTLE) cell was used for detecting the polycyclic aromatic hydrocarbon (PAH) biomarkers 1-hydroxypyrene (1-pyOH) and 1-hydroxypyrene-glucuronide (1-pyOglu) in phosphate buffer and artificial urine. This approach uses selective electrochemical modulation of a fluorescence signal by sequentially oxidizing the analytes in an OTTLE cell to distinguish between their overlapping fluorescence spectra. This technique allows for complete oxidation and signal modulation in approximately 15 min for each analyte; a mixture of 1-pyOH and its glucuronic acid conjugate can be analyzed in 30 min. Calibration curves consisting of the fluorescence change vs analyte concentration for 1-pyOH and 1-pyOglu yielded linear ranges from 10 nM to 1 μM and from 1 nM to 1 μM, respectively. With the use of these results, the calculated limits of detection were determined to be 1×10^{-8} M for 1-pyOH and 9×10^{-11} M for 1-pyOglu.



Here we demonstrate a technique based on spectroelectrochemical sensing in an optically transparent thin layer electrode (OTTLE) cell to detect the PAH biomarkers 1-hydoxypyrene (1-pyOH) and 1-hydroxypyene-glucuronide (1-pyOglu). Thin-layer spectroelectrochemistry in an OTTLE cell couples the ability to change the oxidation state of a compound confined in a thin layer of solution next to an optically transparent electrode (OTE), with the detection capabilities of spectroscopy.[1] The advantages of this technique are rapid and complete analyte electrolysis, available optical window, and selectivity and sensitivity of the spectroelectrochemical approach. With dependence on the thickness of the OTTLE, complete electrolysis can occur within a few minutes. Increasing the thickness of the cell increases the optical path length which provides an increased optical signal. However, this also increases the time required for complete electrolysis of the analytes. OTEs consisting of a thin layer (100−5000 A) of optically transparent and conductive material such as Au, Pt, carbon, or tin-doped indium oxide (ITO) have been used for OTTLEs.[2−10] The optical range of the OTTLE is normally determined by the selection and thickness of the conductive thin film. However, for front-side fluorescence measurements like those made here, the optical window depends only on the choice of the OTTLE cover slide. In this case, a silica window allows for measurements over the visible and UV spectral range.[10]

The pyrene metabolites 1-pyOH and 1-pyOglu have been extensively used as biomarkers for indirect assessment of total polycyclic aromatic hydrocarbon (PAH) exposure.[11−16] These metabolites have been found to be effective biomarkers for PAH exposure assessment because pyrene is a major constituent in virtually all PAH mixtures,[17,18] and its metabolites are readily excreted in urine.[19,20] The metabolism of pyrene involves formation of the phase I metabolite 1-pyOH, which undergoes phase II metabolism with conjugation to glucuronic acid.[21] Assessment of human urine has shown 1-pyOglu accounts for approximately 80% of the pyrene metabolites excreted.[21]

Because of the high concentration of conjugated metabolites, the standard approach for human assessment involves an initial enzymatic or acid hydrolysis of pyrene conjugates to 1-pyOH. The resulting sample of 1-pyOH can then be analyzed by HPLC with fluorescence detection.[11] Though this technique is highly reproducible and offers detection limits in the low microgram/liter range, it requires multiple experimental steps and has an extended analysis time (overnight). A variety of other detection methods have been developed to expedite this process, including HPLC-FL for direct measurement of 1-pyOglu, immunoaffinity chromatography with synchronous fluorescence spectroscopy (SFS), GC/MS, and LC−MS/MS.[22−30] All of these techniques have been shown to offer detection limits within a suitable range for pyrene metabolite detection (1 nM).[31] However, they are all still multistep processes involving an initial separation step for selectivity, followed by detection. Ideally, quantification of these metabolites in a solution mixture would be done in a single analysis that required no dilution or separation step.

Quantification of 1-pyOH and 1-pyOglu without separation is difficult and requires high levels of selectivity and sensitivity due to the metabolites' similar optical properties and the low concentrations commonly found in urine. The spectroelectrochemical technique used here offers a high level of selectivity because it requires an analyte to be electrochemically active at the potential selected and either the analyte or the electrolysis product must absorb or emit light at the selected wavelength. In this case,

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1-pyOH and 1-pyOglu are detected by the change in optical spectra associated with their oxidation. Excellent sensitivity is achieved by using fluorescence detection.

EXPERIMENTAL SECTION

Reagents and Materials. The following chemicals were used as received: ammonium chloride, calcium chloride, citric acid, magnesium sulfate, potassium phosphate dibasic, potassium phosphate monobasic, sodium bicarbonate, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, sodium sulfate, uric acid (all from Fisher Scientific); 1-hydroxypyrene (1-pyOH), 1-hydroxypyrene-glucuronide (1-pyOglu) (both from Toronto Research Chemicals, Inc.); bovine albumin, lactic acid (both from Sigma-Aldrich); urea (J.T. Baker Chemical Co.); and 200 proof ethyl alcohol (Pharmco-AAPER). 1-pyOH and 1-pyOglu solutions were prepared by dissolving appropriate amounts in 0.1 M NaCl/0.05 M pH 7.2 phosphate buffer/30% EtOH (prepared with deionized water from a Barnstead Nanopure system).

The OTTLE cell, which has been previously described,[10] is constructed from a silica slide (1.90 × 1.00 cm), 0.018 cm-thick silicone spacers (Specialty Manufacturing Inc., Pineville, NC), and indium tin oxide (ITO)-coated glass slides (Corning 1737F or 7059, 11−50 Ω/square, 130 nm-thick film on 1.1 mm glass, Thin Film Devices, Anaheim, CA) with dimensions of 4.00 cm × 1.00 cm. Silicone spacers cut to approximately 1.90 cm × 0.20 cm are placed onto the edges of the ITO glass slide and sandwiched between a silica slide and the ITO. Two-part quick setting epoxy (Loctite) is used along the edges of the spacers and allowed to cure for 2 h to hold the components together. The optical path length of the cell is determined by the thickness of the spacers used in cell construction. The exposed ITO above the silica slide is used for electrical contact. For this work, three OTTLE cells were constructed and used, each having an approximate lifetime of 5 h. After 5 h of continuous use, the epoxy would fail from exposure to the ethanol in the solvent and the silica slide would begin to separate from the ITO electrode.

An artificial urine simulant solution containing 2.0 mM citric acid, 1.1 mM lactic acid, 0.4 mM uric acid, 0.5 mM albumin, 25 mM sodium bicarbonate, 170 mM urea, 2.5 mM calcium chloride, 90 mM sodium chloride, 2.0 mM magnesium sulfate, 10 mM sodium sulfate, 7 mM dipotassium hydrogen phosphate, 7 mM potassium dihydrogen phosphate, and 25 mM ammonium chloride was prepared by dissolving appropriate amounts in deionized water.[32] The artificial urine solution was diluted by 30% with EtOH spiked with 5.0×10^{-7} M 1-pyOH and 1.0×10^{-7} M 1-pyOglu.

Instrumentation. For all experiments, the electrochemical cell consisted of a Pt wire auxiliary electrode, a miniature Ag/AgCl reference electrode (Cypress Systems), and an OTTLE. Thin layer cyclic voltammetry was performed on a BAS 100 electrochemical analyzer (Bioanalytical Systems).

Absorbance and emission spectra of 5.0×10^{-5} M 1-pyOH and 1-pyOglu in a 1 cm quartz cuvette were acquired using a Varian Cary 50 Bio UV−visible spectrophotometer and a Varian Cary Eclipse fluorescence spectrophotometer, respectively. Spectroelectrochemical detection of 1-pyOH and 1-pyOglu was performed using the instrumentation arrangement shown in Figure 1, consisting of a laser (325 nm HeCd model 1K3202R-D Kimmon Electric Co.), light control modules (shutter, attenuator, and focusing optics), a monochromator (0.3 m focal length, triple grating turret), a photon-counting phototube (Acton Research Corp.), and a computer and control electronics (NCL and Spectra-Sense software, Acton). Light from the laser was focused onto the polished end of a 6-around-1 silica fiber optic bundle (RoMack, Inc.) with a microscope objective (10×, NA = 0.25, Newport). The laser power was attenuated to 0.5 mW at the OTTLE with a variable neutral density filter (Newport). The sample was exposed to the laser light only during data acquisition to minimize photodegradation. A Bioanalytical Systems Epsilon potentiostat was used for controlled potential electrolysis of 1-pyOH and 1-pyOglu at 750 and 1200 mV, respectively, for 15 min.

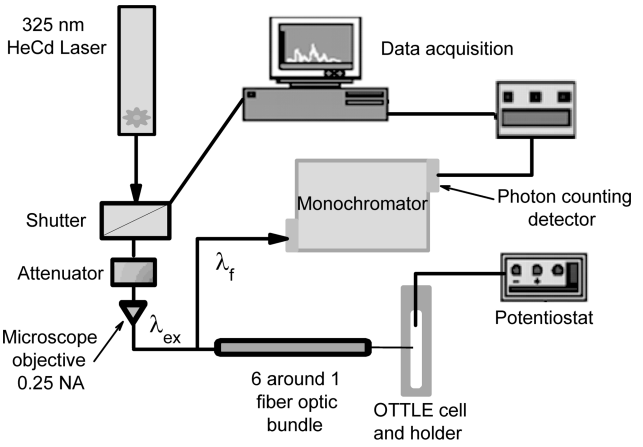

Figure 1. Diagram of the system used for spectroelectrochemical detection.

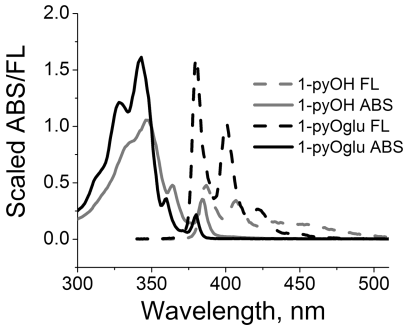

Figure 2. Normalized absorption and fluorescence spectra of 5.0×10^{-5} M 1-pyOH and 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH. Fluorescence excitation at absorption maxima for 1-pyOH and 1-pyOglu (345 and 340 nm, respectively).

RESULTS AND DISCUSSION

The novel sensing method presented here allows for detection of the pyrene metabolites 1-pyOH and 1-pyOglu in a single 30 min analysis. This detection is achieved by using a spectroelectrochemical approach which allows for selective electrochemical modulation of what would otherwise be overlapping absorption and emission spectra of the metabolites in a mixture. At pH 7.2, the absorption and fluorescence spectra largely coincide (Figure 2). The absorption spectra of 1-pyOH and 1-pyOglu at this pH are distinguished only by a 5 nm bathochromic shift for 1-pyOH and by the difference between their molar absorptivities. The fluorescence spectra obtained by

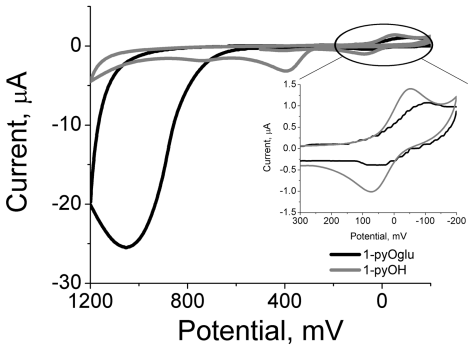

Figure 3. Thin layer cyclic voltammograms of 1.0×10^{-4} M 1-pyOH and 1-pyOglu were obtained from −200 to 1200 mV at a scan rate of 5 mV/s in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH at an ITO electrode. Enlargement from 300 to −200 mV of the redox products from the oxidation of the parent PAH metabolite.

excitation at the wavelength of maximum absorbance for 1-pyOH and 1-pyOglu (345 and 340 nm, respectively) exhibit characteristic pyrene emission peaks (Figure 2). These peaks (vibronic structure) are again separated by only a 5 nm red shift for 1-pyOH along with systematic differences in intensity.

An additional complication is the strong absorbance of 1-pyOH and 1-pyOglu in the UV region (Figure 2). Detection in the UV can be problematic for spectroelectrochemistry because excitation in the UV limits the materials for prisms and waveguides to silica. The previously described OTTLE cell and holder used for this work alleviates this problem by allowing for front side measurements through a silica slide, which is suitable for excitation in the near UV range.[10]

Cyclic Voltammetry of 1-pyOH and 1-pyOglu. The electrochemical oxidation of 1-pyOH at pH 7.2 has been previously studied and shown to involve a complex electrochemical-chemical-electrochemical (ECE) and an electrochemical-electrochemical-chemical-electrochemical (EECE) mechanism.[33] Figure 3 shows similar results for the CV of 1-pyOH at an ITO electrode. With the use of an initial potential of −0.2 V and switching potentials of +1.2 and −0.2 V, two irreversible oxidation peaks at +0.396 and +0.742 V on the initial positive sweep were observed. Following the initial oxidation, a new, quasi-reversible couple is seen with Epc = −0.054 and Epa = +0.074 V.

Figure 3 also shows the CV of 1-pyOglu in the same solution matrix as 1-pyOH at an ITO electrode, which has not been previously investigated. As expected given their structural similarities, the oxidation of 1-pyOglu results in a CV similar to that obtained by the oxidation of 1-pyOH. The voltammagram reveals an initial, irreversible oxidation at +1.050 V leading to a new quasi-reversible couple at Epc = −0.112 and Epa = +0.060 V. The major difference in the CVs is the potential required for the initial oxidation of the metabolites. 1-pyOH is much easier to oxidize than its glucuronic acid conjugate at this pH. As shown in Figure 3, 1-pyOglu requires a potential of approximately +1.050 V vs the +0.742 V required for oxidation of 1-pyOH. While the metabolites are easily distinguishable by electrochemical techniques on the basis of their oxidation potentials, the detection limits for these methods cannot reach the low nanomolar range required for detection of 1-pyOH or 1-pyOglu in practical applications. In order to reach the detection limits required for these metabolites and have the selectivity to distinguish between each in a solution mixture, the potential

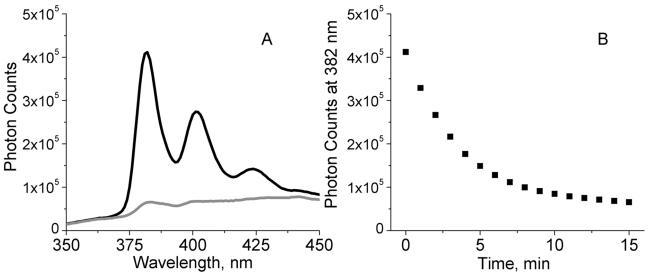

Figure 4. (A) Fluorescence of 1.0×10^{-6} M 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH before and after the application of 1.2 V for 15 min. (B) Fluorescence of 1.0×10^{-6} M 1-pyOglu at 382 nm during oxidation at 1.2 V for 15 min. All potentials vs Ag/AgCl and excitation at 325 nm.

differences can be used in combination with the sensitivity of fluorescence detection.

Spectroelectrochemistry. The spectroelectrochemical technique used here involves measuring a change in the optical signal associated with electrochemical oxidation or reduction of the analyte. Figure 4A shows how the oxidation potential determined by CV can be used to change the fluorescence signal for 1.0×10^{-6} 1-pyOglu over 15 min in an OTTLE cell. The oxidation of 1-pyOglu results in a decrease in fluorescence intensity across the emission spectrum of 1-pyOglu. The greatest change in signal occurs at the fluorescence maximum (382 nm). With the use of this emission wavelength, Figure 4B depicts the change in fluorescence during controlled potential oxidation. At time zero, no potential is applied and this point corresponds to the maximum intensity seen in Figure 4A. The fluorescence decreases over the first 5 min for this concentration and then begins to level off as the electrolysis approaches completion. The point at 15 min in Figure 4B corresponds to the fluorescence counts at 382 nm for the spectrum of lower intensity in Figure 4A.

A similar response (not shown) was obtained for the oxidation of 1-pyOH at 750 mV. This oxidation resulted in the largest change in fluorescence signal at the emission maximum for 1-pyOH of 387 nm. Monitoring this wavelength over the 15 min of potential application resulted in an initial linear portion followed by leveling off of the signal similar to what is shown in Figure 4B. Because of the irreversible nature of the oxidation of 1-pyOglu and 1-pyOH, multiple measurements on a single sample cannot be obtained. This irreversibility results in a single signal modulation for each sample instead of an average of multiple modulations which would decrease the error associated with each measurement by signal averaging.[34]

The dynamic linear range was determined by generating a calibration curve for each analyte in separate solutions. Solutions of 1-pyOH and 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH were excited at 325 nm in an OTTLE cell as previously described. The optical response was monitored at 1 min intervals for 16 min. For the final 15 min of monitoring, a constant potential sufficient for oxidation of the analyte being monitored was applied (750 mV for 1-pyOH and 1200 mV for 1-pyOglu). After 15 min of electrolysis, the final spectrum was subtracted from the initial spectrum (at which no potential was applied). The difference in photon counts at the wavelength of maximum fluorescence for each analyte was used for generation of the calibration curves (382 nm for 1-pyOglu and 387 nm for 1-pyOH).

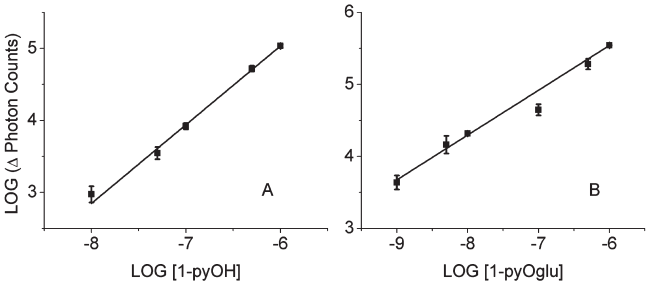

Figure 5. Calibration curves for the change in fluorescence signal of (A) 1-pyOH in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH at 387 nm with 750 mV over 15 min and (B) 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH at 382 nm with 1200 mV over 15 min. Each point is the average of a minimum of three trials. Excitation at 325 nm and potentials vs Ag/AgCl.

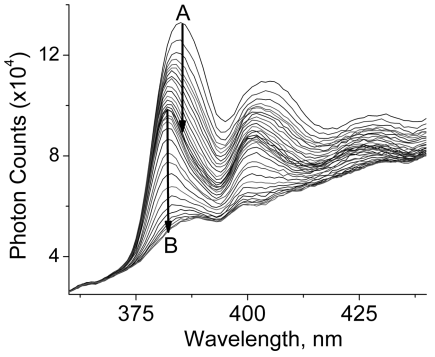

Figure 6. Fluorescence modulation of a mixture of 5.0×10^{-7} M 1-pyOH (A, modulation at 750 mV over 15 min) and 1.0×10^{-7} M 1-pyOglu (B, modulation at 1200 mV for 15 min). The change in signal for each analyte is measured at their respective fluorescent λ_{max} following the application of potential sufficient for oxidation. Each scan is 1 min. Excitation at 325 nm and all potentials vs Ag/AgCl.

Following each trial, the OTTLE was washed twice with 100% EtOH. The small error associated with each randomly introduced concentration in Figure 5A,B confirms that this is an appropriate cleaning method that results in little or no carryover from trial to trial.

The calibration curve generated for 1-pyOH is shown in Figure 5A. A linear relationship between the log of the difference in photon counts, Δ, and the log of [1-pyOH] in sample solutions ranging from 10 nM to 1 μM was observed. The equation for the linear range was $y = (1.09 \pm 0.03)x + (11.59 \pm 0.19)$ (R^2 = 0.997). The detection limit for 1-pyOH was calculated to be 1×10^{-8} M using the previously described method of three times the experimentally determined standard deviation of the blank (261 counts) and the slope of the linear equation obtained from the calibration curve.[35]

Figure 5B shows the calibration curve generated for 1-pyOglu. Again, a linear relationship between the log of Δ and the log of [1-pyOglu] in sample solutions ranging from 1 nM to 1 μM was observed. The equation for the linear range was $y = (0.62 \pm 0.02)x + (9.28 \pm 0.15)$ (R^2 = 0.993). The detection limit for 1-pyOglu was calculated to be 9×10^{-11} M. The lower limit of detection for 1-pyOglu compared to 1-pyOH was expected due to the approximately 3−5× larger quantum yield of 1-pyOglu.[14] A contributing factor was the decreased standard deviation of the blank at 382 nm (238 counts). These two factors allow for the calculated detection limit of 1-pyOglu to be lower than the expected physiological range for individuals exposed to PAHs.

The difference in redox potentials enables the fluorescence change associated with each analyte in a mixture to be measured. Figure 6 shows the sequential fluorescence modulation of 5.0×10^{-7} M 1-pyOH (A) and 1.0×10^{-7} M 1-pyOglu (B) in a single analysis. The spectrum of highest intensity in Figure 6 is attributed to the unoxidized 1-pyOH and 1-pyOglu in solution. It is clear from this spectrum that quantifying the concentration of analytes by fluorescence alone is difficult. However, by a sequential change in the potential from 750 to 1200 mV, the fluorescence signal associated with each analyte can be selectively modulated. A potential of 750 mV was first applied to the mixture for 15 min, resulting in the oxidation of 1-pyOH (Figure 6A). As with the calibration curve, the change in fluorescence was determined by subtracting the final spectrum after 15 min of electrolysis from the initial spectrum at which no potential was applied. This calculation resulted in a modulation amplitude of 48 000 counts, which correlates nicely with the 52 000 ± 5 000 counts obtained from the calibration curve obtained with the single analyte. Following the 15 min oxidation at 750 mV, the potential was stepped to 1200 mV, resulting in the oxidation of 1-pyOglu (Figure 6B). The change in fluorescence signal associated with this was 46 000 counts, which again falls well within a standard deviation of the mean (44 000 ± 8 000 counts) obtained from the calibration curve.

It is important to note that complete oxidation of 1-pyOH is required in the initial potential step because any unoxidized 1-pyOH will oxidize during the sequential oxidation step. Oxidation of 1-pyOH during the second sequential step will result in a fluorescence modulation which would be incorrectly associated with 1-pyOglu concentration due to the overlap in fluorescence spectra. The complete oxidation of 1-pyOH can clearly be seen in Figure 6 by the leveling off and ultimately coinciding spectra at 387 nm, near the end of the initial oxidation.

Similar plots were obtained for 5.0×10^{-7} M 1-pyOH and 1.0×10^{-7} M 1-pyOglu in an artificial urine solution. Electrochemical modulation using the same procedure described for the buffered solution resulted in a signal modulation of 54 000 ± < 1 000 for 1-pyOH and 40 000 ± 2 000 for 1-pyOglu. Both of these values fall within the expected range determined by the single analyte calibration solutions (52 000 ± 5 000 for 1-pyOH and 44 000 ± 8 000 for 1-pyOglu).

CONCLUSIONS

The spectroelectrochemical technique demonstrated here was shown to be capable of quantitatively distinguishing between two analytes with overlapping fluorescence spectra without the need for an initial separation step. The analytes selected here (1-pyOH and 1-pyOglu) are a good model system for this detection method because of their near identical absorbance and fluorescent spectra and the large difference in their initial oxidation potentials. This approach could be used for other analytes and more complex mixtures as long as the analytes' redox potentials are sufficiently separated.

In this work spectroelectrochemical sensing was used for detection of a mixture of 1-pyOH and 1-pyOglu in a single 30 min analysis in a phosphate buffer and an artificial urine solution. This approach provided a calculated limit of detection for 1-pyOglu within the required range for physiological measurements in

urine to evaluate exposure to PAHs. However, the 10 nM detection limit obtained for 1-pyOH is adequate for some physiological measurements but not down to the lowest exposure levels of interest. A lower limit of detection can be obtained by increasing the thickness of the OTTLE by simply increasing the thickness of the spacers used for cell construction. The increased optical path length would require longer electrolysis times but would result in larger changes in fluorescence for each analyte concentration.

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