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Analysis of Phthalate Esters in Mammalian Cell Culture Using a Microfluidic Channel Coupled with an Electrochemical Sensor

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ABSTRACT: An analytical tool to monitor trace phthalate was developed using a microfluidic channel device coupled with a novel electrochemical biosensor. At first, the electrochemical sensor was constructed with biomimetic layers to reveal a large hydrogen over potential by controlling the surface charge and hydrophobicity through assembling with a lipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and a cationic molecule (toluidine blue O) bonded to a conductive polymer. The modified electrode possessing a highly negative polarization potential (approximately -1.8 V vs. Ag/AgCl) can uptake sparingly soluble phthalate ester (PEs) compounds in aqueous media. Each sensor probe material was characterized employing SEM, AFM, XPS, QCM, TEM, UV-visible, and impedance spectroscopy. The microfluidic channel is used firstly to concentrate and separate trace amount of phthalates, then the sensor probe is installed at the end of channel. Experimental variables affecting the PEs analysis were assessed and optimized in terms of biomimetic layer composition and analytical conditions. The linear dynamic range and detection limits of the PEs were 0.15 nM - 10.0 μ M and ~12.5 pM with relative standard deviations <5%. The proposed method was applied to evaluate the effect of endocrine disruptors on mammalian kidney cell, where the cell samples show in-taking percentages between 1.8 and 7.0% to the total PEs according to the incubation time.

Phthalate esters (PEs) widely used as plasticizers in polymers omnipresent in the environment and exhibit toxic, carcinogenic, mutagenic, and teratogenic effects on animals.¹⁻³ Recent publications on the endocrine disrupting properties of PEs have disclosed their hazardous effects over a long-term exposure to the environment from various perspectives, including their multifarious threats on human health and wellbeing.⁴ Most countries in the world, nowadays, have classified some of the PEs as high-priority pollutants due to the decline in the male ratio, pre-mature breast development, and breast cancer.⁵⁻⁸ A maximum admissible concentration (MAC) of $3 \mu\text{g L}^{-1}$, for di (2-ethylhexyl) phthalate (DEHP) in water is established by the U.S. Environmental Protection Agency (EPA).⁹ DEHP is the most widely used PE and it encompasses a quarter of the total production of plasticizers.¹⁰ PE's are known to induce toxic effects in the mammals,¹¹ and they are known to disrupt the maturation of Leydig and Sertoli cells leading to a reduction in germ cells in the malformed seminiferous tubules in adulthood as well as an increased incidence of multinucleated germ cells.¹² Hence, monitoring the traces of these compounds present inside the cultured cells as a result of their cellular penetration is crucial to diagnose the status of human health and environmental control of the PE's.

Mostly, the extraction and pre-concentration steps are performed in advance to analyze trace levels of PEs in water samples, conventional employing methods such as solid-phase extraction and liquid-liquid extraction.¹³⁻¹⁵ The analysis of pre-concentrated PEs can be achieved using sensitive detection methods, such as mass spectrometry (GC-MS and LC-MS),^{13,16} however, these methods are time-consuming and expensive. One of the promising alternatives to overcome these limitations is the on-line sample pre-concentration^{14,17} using microfluidic devices.^{18,19} Trace analysis of endocrine disruptors (EDs) was introduced using microfluidic device with high sensitivity and speed.^{19,20} Thus, we try to monitor the trace PEs with a microfluidic channel coupled with an electrochemical biosensor in the present study.

To date, no electrochemical method using conventional solid electrode has been reported for analysis of PEs due to the highly negative reduction potential. We therefore tried to develop a modified electrode that exhibited an extremely negative polarization potential in aqueous media without hydrogen evolution (approximately -1.8 V vs. Ag/AgCl) on the surface. To develop the modified electrode with hydrophobic property, we firstly modified the surface with a conducting polymer, phospholipid, and an organic molecule to provide a hydrophobic and positively charged biomimetic surface layer to interact with negatively charged target molecules. Conducting polymers have received considerable attention as sensor substrate materials for signal amplification and surface modification to confer stability upon the electrode.²¹⁻²³ Thus, it is employed as the substrate to form a biomimetic layer for use as an electrode material that is highly stable, sensitive, and selective to PEs. To enhance the sensitivity of the sensor probe, gold nanoparticles (AuNPs) was used because of their high electrocatalytic activity, ease of chemical modification, size-dependent electrical properties, and structural and functional compatibility with biomolecules.¹⁸

In the present study, a microfluidic device that includes double pre-concentration channels along with micellar electro kinetic separation, was fabricated in conjugation with an electrochemical sensor comprising a biomimetic layer for the analysis of PEs (dibutyl phthalate (DBP), DEHP, dicyclohexyl phthalate (DCHP), diethyl phthalate (DEP), and benzyl butyl phthalate (BBP)). The sensor probe was modified with toluidine blue O (TBO) along with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). Various parameters affecting the performance, including the mole ratio of DOPE and TBO, the detection potential, the pH, and the running buffer concentration were optimized. The method was tested for real samples like manicure and PVC, which are used in everyday life. We also utilized the mammalian non-cancerous cell lysates to study the percentage of intake and the

minimal concentration of the PE's affecting the growth of the cells in culture using the proposed system.

■ MATERIALS AND METHODS

Chemicals and Instruments. Standard PE samples (DEHP, DBP, DEP, DCHP, and BBP), $\text{HAuClO}_4 \cdot 3\text{H}_2\text{O}$ ($\geq 99.9\%$), HCl (37%), TBO, and DOPE, and fetal bovine serum (FBS). Vero mammalian kidney cell sample was purchased from the Korean Cell Line Bank (S. Korea). TBAP (Tetrabutylammonium perchlorate) was purchased from Fluka (USA). A terthiophene monomer TTBA (2,2':5',2''-terthiophene-3'-(*p*-benzoic acid)) was prepared according to the previously reported procedure.²⁴ The phosphate buffer solution (PBS, pH 7.4) was prepared using NaH_2PO_4 and Na_2HPO_4 mixtures. Britton-Robinson buffer (pH 10.0) was used as a stacking buffer in field-amplified sample stacking (FASS) and field-amplified sample injection (FAI) step with electrochemical detection. All the aqueous solutions were prepared using double distilled water obtained from the Milli-Q water purifying system (18 M Ω cm).

The electrochemical measurements were taken employing a three electrode system, where the modified glassy carbon (GC, dia. 3.0 mm) was used as the working, Ag/AgCl (sat. KCl) was used as the reference, and Pt wire was used as the auxiliary electrodes. The electrochemical experiments including voltammetry, impedance spectroscopy, and QCM were performed with several instruments (see detail Supporting Information).

Preparation of Sensor Probe. The construction of the biomimetic layer is shown in Scheme 1(A). A layer of the polymer of the TTBA monomer was formed on the AuNPs/GCE surface through electrochemical polymerization of 1.0 mM solution of the monomer TTBA in a 0.1 M TBAP/ CH_2Cl_2 by a single potential cycling from 0.0 to +1.4 V at a scan rate of 100.0 mV/s (inset Scheme 1(A)) (see detail Supporting Information).

Fabrication of Microfluidic Channel. The detailed fabrication of microfluidic channel is

explained in the Supporting Information. The microfluidic system consists of parallel FASS and FASI channels for the pre-concentration of sample, in conjunction with a separation channel. The microfluidic device was fabricated for the pre-concentration and the separation of analytes as shown in Scheme 1(B). To confirm the performance of the separation method, the standard PEs concentrations in the samples were measured using a GC/MS.

Preparation of Real PEs Samples. PEs were extracted from manicure and PVC samples using the standard method recommended by the Ministry of Food and Drug Safety (MFDS). Mammalian kidney epithelial cells (Vero) were cultured in a high glucose Dulbecco's modified eagles medium (DMEM) supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin (100 units/mL) at 37 °C under 5% CO₂ and 95% humidified conditions (details in the Supporting Information).

■ RESULTS AND DISCUSSION

Fabrication of Sensing Probe. To confirm the sensor probe fabrication, each layer of the sensor was characterized using SEM, AFM, XPS, QCM, and impedance spectroscopy. For the first layer of AuNPs on the GC, the SEM image was obtained for the AuNP layer that shows the formation of 20.0 ± 2.5 nm AuNPs (Supporting Information, Figure S1(A)). Image of the polyTTBA coated AuNPs modified surface shows a homogeneous polymer layer, where no NPs were observed due to the covering of the surface by the polymer (Supporting Information, Figure S1(B)). After that, the assembling of the biomimetic layer (DOPE and TBO) on the polyTTBA/AuNPs were separately confirmed by taking AFM images of the surface for each step. Figure 1 shows the three- and two-dimension AFM images obtained for (A) polyTTBA, (B) DOPE/polyTTBA, and (C) DOPE + TBO/polyTTBA coated on the highly oriented pyrolytic graphite electrode (HOPGE) surfaces. The surface of the polyTTBA layer exhibited a plane and uniform conducting polymer layer composed of particles of 30.5

± 5.5 nm diameter. The DOPE and TBO immobilized onto the polyTTBA layer through covalent bonding can be clearly seen in the AFM image obtained for DOPE/polyTTBA and DOPE + TBO/polyTTBA. The width of the lipid (DOPE) was found to be ~2 nm, which finely covers the sensor probe. The difference in the root mean square (RMS) surface roughness between the polyTTBA and DOPE/polyTTBA films is small, as is depicted in Figure 1, which were 0.55 and 0.48 nm. On the other hand, the RMS roughness of the DOPE + TBO/polyTTBA layer is 0.86 nm.

The covalent bond formation between polyTTBA and lipid including TBO on sensor surfaces were characterized using XPS, where the spectra were calibrated employing a C1s peak at 284.6 eV as an internal standard and measured after etching the surface with Ar ion for 50.0 s. Supporting Information, Figure S2 shows the de-convoluted XPS spectra for (A) C1s, (B) N1s, (C) S2p, (D) Au4f, (E) O1s, and (F) Cl2p peaks for (I) polyTTBA, (II) DOPE/polyTTBA, (III) TBO/polyTTBA, and (IV) DOPE + TBO/polyTTBA on AuNPs/GCE surfaces. Two peaks at 284.6 and 288.3 eV were observed in the C1s spectrum, corresponding to C-S, C-C, C-H, or C=C bonds and C-O, C=O, or O-C-O bonds, respectively. A clear peak at 399.6 eV was observed in the N1s spectrum, corresponding to the C-N bond because of the amide bond formation between polyTTBA and TBO or DOPE, whereas no peak appears for the nitrogen atom at polyTTBA/AuNPs layer. In addition, a peak was observed at 163.7 eV for the S2p spectrum, corresponding to the C-S bond from the polyTTBA molecule. The Au4f spectrum exhibits a peak at 83.7 eV for all of the AuNP-containing surfaces. Two peaks corresponding to C=O and C-O appear at 532.1 and 533.2 eV in the O1s spectrum of the polyTTBA layer. After the DOPE is immobilized, an additional peak appears at 534.1 eV, which corresponds to P=O or P-O because of the presence of the lipid's phosphatidyl groups.²⁵ The Cl2p spectrum exhibits a peak at 200.0 eV,²⁶

corresponding to the TBO layer. The XPS results show that all the layers are successfully formed on the electrode surface.

QCM experiments were also performed to estimate the amounts of (a) DOPE, (b) TBO, and (c) DOPE + TBO immobilized on the polyTTBA film based on the frequency change (Figure 2(A)). In the case of DOPE, TBO, and DOPE + TBO immobilization, a steady state in the frequency is achieved after 32, 43, and 54 min, with overall frequency changes (Δf) of 204.1, 185.7, and 259.1 kHz, respectively. The immobilized amounts of DOPE, TBO, and DOPE + TBO were 224.4 ng (1.5×10^{-9} mol/cm²), 204.2 ng (3.4×10^{-9} mol/cm²), and 284.8 ng, respectively, using a previously defined equation.²⁵ The above results demonstrate that DOPE and TBO are successfully oriented onto the polymer layer.

Electrochemical Behavior of Sensor Probe. The electrochemical properties of sensor probe and the response to the PE reduction in the aqueous solution were investigated with impedance spectroscopy and LSV. Impedance spectroscopy was employed to investigate the surface charge and conductivity of each layer of the sensor probe. Figure 2(B) shows the Nyquist plots obtained for AuNPs/GC, polyTTBA/AuNPs/GC, DOPE/polyTTBA/AuNPs/GC, TBO/polyTTBA/AuNPs/GC, and DOPE + TBO/polyTTBA/AuNPs/GC electrodes in a 0.1 M Britton-Robinson buffer solution containing acetonitrile (10 v/v %). The Nyquist plots show an increase in the resistance of the electrode surface due to the immobilization of DOPE and TBO onto the polyTTBA/AuNPs/GCE. The experimental impedance data for polyTTBA/AuNPs/GCE were simulated using a simple Randle Circuit. Supporting Information, Table S1 shows values for CPE1, CPE2, R_s , R_p1 , R_p2 , and W which were determined by fitting experimental data to the equivalent circuit using the Zview2 software. In the equivalent circuit, R_s represents the solution resistance, and the polarization resistances are represented by R_p1 and R_p2 , W represents the Warburg element, and the constant-phase elements are denoted as CPE1 and

CPE2. From this analysis, the polyTTBA/AuNPs/GCE exhibited a low R_{p1} value of 384 Ω cm^2 . After DOPE, TBO, and DOPE + TBO were immobilized, the R_{p1} and R_{p2} values were 847, 793, and 1192 and 4688, 3948, and 6582 Ω cm^2 which are higher than the values of R_{p1} and R_{p2} for the polyTTBA/AuNPs/GCE surface, and indicating that the molecules impeded on the electron-transfer reaction in the layer. The analytical performance of DOPE + TBO/polyTTBA/AuNPs/GC sensor surface was evaluated under the previously determined optimum conditions. The sensor was immersed in a blank buffer solution (pH 10.0) containing acetonitrile, and LSVs recorded between +0.5 and -1.7 V (vs. Ag/AgCl) indicated no redox peak (not shown), the test solution was purged using nitrogen gas to avoid any interference from the reduction of dissolved oxygen during the PEs analysis. Otherwise, when the sensor surface was dipped into the buffer solution with 1.0 mM mixed PEs, a peak was observed at -1.57 V because of the reduction of PEs on the DOPE and TBO layer, which demonstrates that the DOPE + TBO modified electrodes responded well to phthalate (Figure 2(C)). To examine the responses of different types of PEs individually, LSVs were recorded for DBP, DEHP, DCHP, DEP, and BBP, which revealed reduction peaks at similar potentials on the DOPE + TBO/polyTTBA/AuNPs as shown in Figure 2(D). The reduction peaks of DBP, DEHP, DCHP, DEP, and BBP appeared at -1.60, -1.62, -1.59, -1.58, and -1.61 V, respectively, vs. Ag/AgCl electrode. For the complete electrochemical reduction of test compounds, we selected a -50.0 mV more negative potential for chronoamperometric experiments to detect them in a microfluidic channel. Thus, the chronoamperometric response was obtained at the applied potential of -1.7 V.

Optimization of Detection Variables. In order to optimize the detection conditions using the sensor, the current response was observed as a function of the ratio of (A) DOPE:TBO (v/v %), (B) detection potential, (C) pH, and (D) running buffer concentration (Supporting Information, Figure S3). First, the effect of the relative amounts of DOPE:TBO on the sensor

response was investigated for ratios between 1:2 and 5:1 (Supporting Information, Figure S3(A)). The current response showed a gradual increase as the ratio increased from 1:2 to 2:1, the response showed no further increase after that. Hence, the ratio of 2:1 was selected as the optimum content ratio for analytical performance. Changes in the electrochemical reduction of phthalates as an effect of the applied potential was investigated between -1.5 and -1.8 V (Supporting Information, Figure S3(B)). A gradual increase in the reduction current of the phthalates was observed from -1.5 to -1.7 V, but no significant increase in the reduction current is seen with further increase in the applied potential. Thus, -1.7 V was used as the optimum detection potential in subsequent experiments. The current response showed an increase as the pH of the system was increased from 6.0 to 10.0 and showed a decrease when the pH was further increased from 10.0 to 12.0 as shown in the Supporting Information, Figure S3(C). The maximum current response was observed between pH 9.0 and 12.0, so pH 10.0 was chosen as the optimized detection pH. The response current increased as the running buffer concentration increased from 5.0 to 20.0 mM, and the response current decreased as the concentration increased from 20.0 to 30.0 mM due to the deactivation of the DOPE and TBO. Thus, 20.0 mM was selected as the optimum running buffer concentration in subsequent experiments (Supporting Information, Figure S3(D)).

Stability of the Sensor. The sensor retained 96.5% of its initial response, when it was stored at room temperature (in a vacuum) for four months. In addition, the stability of the sensor for multiple uses was evaluated by repeatedly using the same sensor probe in the PE-containing solution. The sensor lost only 2.7% of its initial response after approximately 45 continuous measurements. This superior stability of the DOPE + TBO/polyTTBA/AuNPs sensor probe might be ascribed to its stable biomimetic layer formation. After using the sensor probe continuously for two months, 87% of its initial response was retained.

Separation Efficiency of the Microfluidic Channel. The procedure for separation, pre-

concentration, and detection of PEs in the microfluidic channel was described in the Supporting Information. The behavior of the sensor (DOPE + TBO/polyTTBA/AuNPs) for 1.0 μM test species in the separation channel, was examined using a 10.0 mM Britton-Robinson buffer (pH 10.0) containing 20.0 mM SDS in acetonitrile. The separation was completed within 155.0 s for all test phthalates as shown in Supporting Information, Figure S4. DBP, DEHP, DCHP, DEP, and BBP showed migration times of 91.4 (± 2.5), 125.3 (± 2.8), 151.7 (± 5.2), 77.7 (± 4.8), and 104.63 (± 3.5) s, respectively. The half-peak widths ($W_{1/2}$, the peak width at maximum half point), resolution ($R_s = 2t_R/(W_1 + W_2)$), and corresponding separation efficiency (the number of the theoretical plates, $N = 5.54 (t_R/W_{1/2})^2$ of DBP, DEHP, DCHP, DEP, and BBP were ~ 9260 , ~ 5200 , ~ 18800 , ~ 15200 , and ~ 13500 , respectively.

Analytical Performance of Microfluidic Channels. In order to study the effects of PEs at a cellular level, normal mammalian cells were subjected to *in vitro* PE analysis. The pre-concentration experiment was carried out using two on-line channels that included FASI connected to FASS. Figure 3(A) shows the electropherograms obtained using (a) only FASI, and (b) FASS and FASI together. In case of (b), using the nanoparticles provided additional active surface area and interaction sites with which the solutes could interact with each other. The AuNPs were prepared using citrate reduction method and explained in detail in the Supporting Information. An absorption band at 520 nm in the UV-visible spectrum (Supporting Information, Figure S5(A)) and TEM image (Supporting Information, Figure S5(B)) confirmed that the particles size in this solution was ~ 3.5 nm. The desired dilutions were prepared daily using appropriate buffer solutions. The AuNPs present in the running buffer significantly affected the apparent mobility (μ_{ap}) of the solute, as well as the electro-osmotic mobility (μ_{eo}) of the running buffer. Further, to enhance the selectivity in separation and sensitivity during detection of the pre-concentration method, the separation and pre-

concentration buffers were modified with charge-stabilized AuNPs. AuNPs were added to the low-pH BGE buffer in the FASI pre-concentration step. Here, the aggregation of the AuNP's was avoided by the surrounding of anion surfactant and SDS. The stacking efficiency of the buffer showed an enhancement due to the increase in the relative conductivity of the stacking buffer as a result of the presence of AuNPs, along with the enhancement in the adsorption of PE's onto the AuNPs surfaces and minimization of the PE adsorption on the channel wall. The concentration of the test compound that required the samples to be pre-concentrated with FASS and FASI together and with FASI only was 1.0 nM. The test compounds were pre-concentrated using FASS and FASI together and FASI only and were detected within the same migration time. The final enhancement of the sensitivity using FASS and FASI was ~7.5-fold, considering the dilution ratio of the sample solution and the enhancement of the current response. Comparing the two electropherograms (Supporting Information, Figure S4) and Figure 3(A(b))), we can conclude that the pre-concentration factor was enhanced approximately ~2500-fold by using the FASI and FASS steps. Supporting Information, Figure S6 shows the electropherograms recorded for the sensor probe at different concentrations of PEs. The calibration curve was obtained by plotting the peak area against the sample concentration, as shown in Figure 3(B). At the optimized conditions, the response precision, dynamic range, concentration enhancement factor, and detection limit were calculated and are summarized in Supporting Information, Table S2. The experiments were repeated several times to get the recurrent values. The dynamic ranges of the PEs are determined to range from 0.15 nM to 10.0 μ M with detection limits between ~12.5 and ~35.2 pM, which is far better than the previous works for the separation and detection of PEs are based on the GC/MS, LC/MS, and HPLC coupled with solid phase micro-extraction (SPME) or liquid-liquid extraction with the detection limits of 0.006 and 0.17 μ g L⁻¹ (SPME-GC/MS),²⁷ 1.5 to 3.0 μ g L⁻¹ (liquid-liquid extraction/HPLC),²⁸ and 0.1 to 4.0 ng mL⁻¹

(SPME/HPLC).²⁹

Analysis of Manicure and PVC. The real samples were collected from (a) manicure and (b) PVC, and the concentrations of their PE content were determined using the SAM (standard addition method) (Figure 3(C)). The untreated buffer and low-pH BGE buffer solutions were filtered by the Millipore filters before adding AuNPs to them. Inset Figure 3(C) shows the electropherograms for the manicure and PVC samples. In the manicure samples, the DEP peak was separated from the others and was detected with the same migration times as with the standard sample (inset Figure 3(C(a))). In the PVC samples, DEHP peak was separated and detected at the same separation time as for the standard sample (inset Figure 3(C(b))). Individual standard phthalates ((II) 10.0, (III) 25.0, and (IV) 50.0 nM) are spiked into the real samples (50.0 μ L (a-I) manicure and (b-I) PVC samples) to confirm the peaks showed in Figure 3(C). The actual DEP and DEHP contents of the manicure and PVC samples were calculated to be 12.58 ($\pm$ 1.16) and 30.05 ($\pm$ 2.34) nM, respectively (Supporting Information, Table S3). The results were compared with the results from a commercial PE analytical instrument using GC/MSD, which showed similar values for phthalate contents in the manicure and PVC samples of 12.54 ($\pm$ 1.02) and 30.07 ($\pm$ 2.15) nM, respectively. The average range recoveries ($n = 3$) of 92 and 96% are achieved for all the PE compounds in the manicure and PVC plastics, where 9.2 and 7.8% of the coefficients of variation were obtained, respectively. However, there are some additional peaks apart from the targets, in both the electropherograms, probably due to the trace phthalate compounds or any other slightly cathodic compounds that may be present in the PE samples being pre-concentrated during the process and detected at -1.7 V using the proposed sensor.

Analysis of Mammalian Cells. The non-cancerous mammalian kidney cells (Vero), cultured in a high glucose DME medium along with different concentrations (50.0, 250.0, and 500.0 nM) of five different PEs were observed under an optical microscope at different

intervals of time, at 6th, 12th, and 24th hr to check for any changes in the cell condition as shown in Figure 4(A). After 6 hrs of incubation in 50.0 nM solution it shows no notable change (Figure 4(A(b))) as compared to the control culture without any PE (Figure 4(A(a)) and inset of Figure 4(A(a))), whereas, small granular materials were observed in the cytoplasm of the cells after 12 and 24 hrs of incubation ((c) and (d)). The 250.0 and 500.0 nM concentrations shows similar granular materials in cell cytoplasm with increased intensity (Figure 4(A(e)) and (h)). As the incubation time and the concentration of the PEs increased the granules in the cytoplasm of the cells were darker and more prominent in nature (Figure 4(A(f) and (g)) and ((i) and (j))). During the experiment it was noticed that the growth rate of the cells was considerably decreased as compared to the control culture, which may be because of the toxic effect of the PEs on the cells. It was also observed that the PEs have a degradative effect on the polystyrene material of the cell culture vessel, which was precoated with the extracellular matrix to facilitate the cell attachment and growth. Finally, after 24 hrs of incubation, the cells were removed from culture and subjected for sample analysis in a microfluidic channel coupled with an electrochemical sensor to further confirm the intake of PEs by the cells in culture.

The samples collected from the cells were used to determine the PE concentration by SAM. Electropherograms for the cell lysate samples of different concentrations ((a) 50.0, (b) 250.0, and (c) 500.0 nM) and incubation times ((I) 6, (II) 12, and (III) 24 hrs) are obtained as shown in Figure 4(B). A small separation peak for DCHP is observed with the same migration time in the 6 hr incubated sample with 50.0 nM concentration of PEs, corresponding to 0.91 ± 0.10 nM (1.8%) of the total DCHP added, but no peaks were observed for the other PEs. As the incubation time increased to 12 and 24 hrs, the separation peaks for DEP and DCHP relating to 0.85 ± 0.08 nM (1.7%) and 1.40 ± 0.14 nM (2.8%) and DEP, DBP, BBP, and DCHP corresponding to 2.9, 1.5, 2.5, and 3.9%, respectively, of the PEs taken in by the cells were

observed, with respective increase in the separation peaks as shown in Figure 4(B(a)). Similarly, in the samples with higher concentration (250.0 and 500.0 nM) of PEs, the electropherograms show increased separation peak current and amount of PEs intake by cells for the five species of PEs with the increased incubation times as shown in Figure 4(B(a-c)). Representatively, the increase in the amount of PEs taken by cells can be explained with help of DCHP, which shows the highest separation peak current. The concentration of DCHP after 24 hrs of incubation at 250.0 nM is 4.4%, whereas, the concentration of the same was 7.0% after 24 hrs of incubation at 500.0 nM. The concentration ranges below 50.0 nM could not be detected because of lack of intake of PEs by the cells, and the concentrations above 500.0 nM poses a difficulty to culture the cells by degrading the inner surface of the culture flask. The mammalian kidney cells show a percentage of intakes between 1.8 and 7.0% to the total of particular PEs in culture depending on the time of incubation.

The results shown in the electropherograms are in complete agreement with the microscopic images (Figure 4(A)) obtained earlier during the culture. The concentrations of the PEs taken in by the cultured cells were determined by the SAM and the results were tabulated in Supporting Information, Table S4. When applying a pre-concentration method a relatively unstable baseline is often observed for the analysis of real samples. The interference of the commonly present electroactive compounds in the real samples was examined, but there were no unknown reduction peaks in the electropherograms obtained for the real samples due to the electrochemical reduction of aqueous sample solutions at the extremely negative potentials.

■ CONCLUSIONS

The sensor probe having the high overvoltage to hydrogen evolution was successfully developed by immobilizing the lipid (DOPE) and cationic TBO to be a hydrophobic and

positively charged biomimetic surface, which amplified the selectivity and the sensitivity of sparingly reducible organic compounds in aqueous media. The trace quantities of the phthalate EDs, DEP, DBP, BBP, DEHP, and DCHP were successfully pre-concentrated, separated, and detected employing microchip electrophoresis coupled with electrochemical detection using the biomimetic sensor probe. The migration times of DBP, DEHP, DCHP, DEP, and BBP were $91.4 (\pm 2.5)$, $125.3 (\pm 2.8)$, $151.7 (\pm 5.2)$, $77.7 (\pm 4.8)$, and $104.63 (\pm 3.5)$ s, respectively, and the separation was performed within 155 s. The detection limits for all the analytes were determined to be between ~ 12.5 and ~ 35.2 pM and the proposed method was successfully performed for the analysis of real samples including manicure, PVC plastics, and mammalian cells, which may enable the development of analysis strategies of some sex related disorders. In addition, this novel method is potentially applicable to the drug screening and biomedicine as well as to monitor various EDs in biological and environmental samples because of its excellent performance.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information available as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Figure captions

Scheme 1. (A) Schematic representation of the sensor fabrication. (B) Schematic diagram of chemical structures of PEs and the microfluidic device.

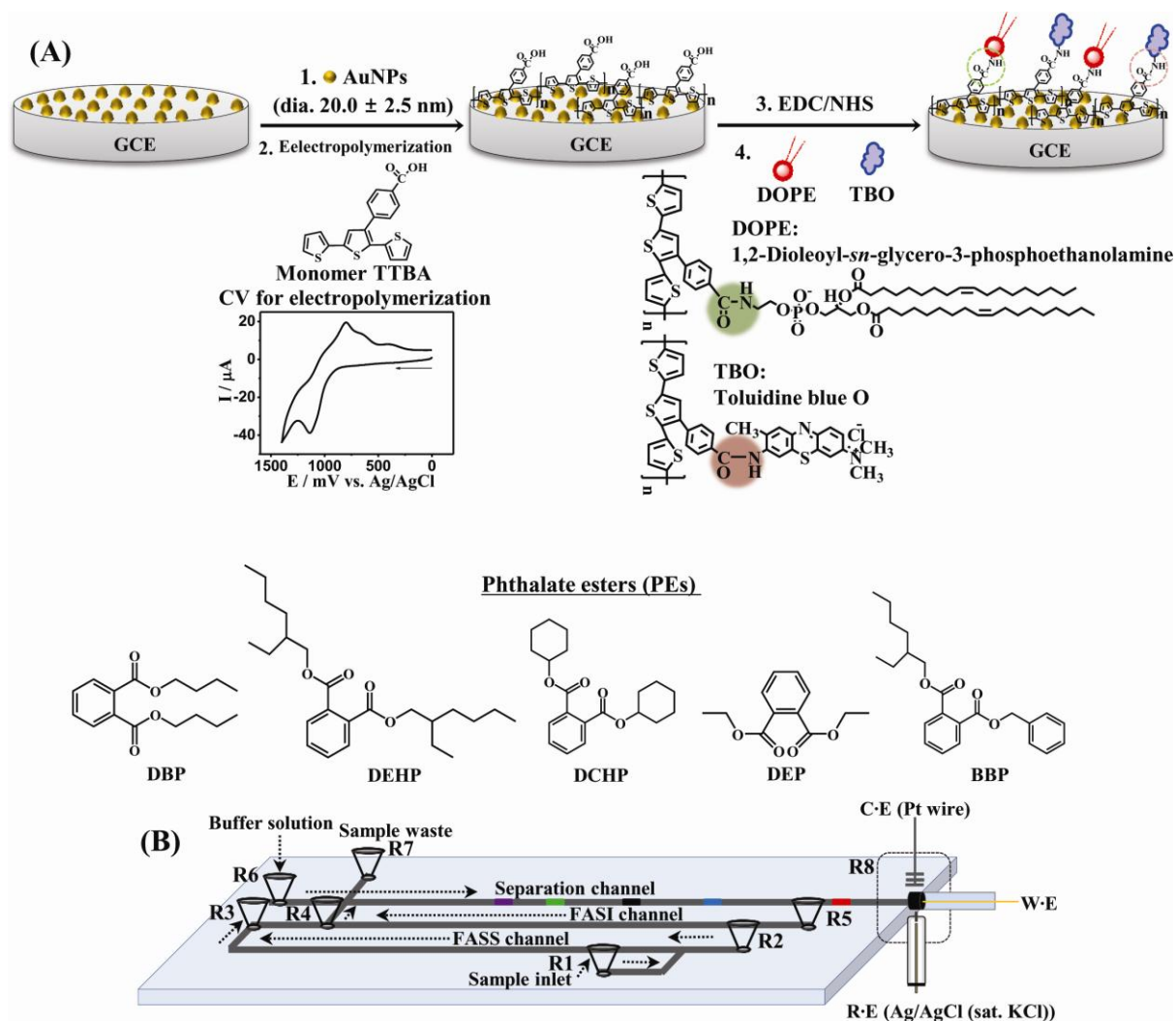
Figure 1. Tapping mode AFM images of the (A) polyTTBA, (B) DOPE/polyTTBA, and (C) DOPE + TBO/polyTTBA films; images size is 500.0 nm x 500.0 nm.

Figure 2. (A) Frequency changes during the immobilization of (a) DOPE, (b) TBO, and (c) DOPE + TBO onto polyTTBA on an Au electrode. (B) Nyquist plots obtained for (a) AuNPs/GC, (b) polyTTBA/AuNPs/GC, (c) DOPE/polyTTBA/AuNPs/GC, (d) TBO/polyTTBA/AuNPs/GC, and (e) DOPE + TBO/polyTTBA/AuNPs/GC electrodes in a 0.1 M Britton-Robinson buffer solution (pH 10.0) containing acetonitrile (10 v/v %). (C) LSVs recorded for the AuNPs/GC, polyTTBA/AuNPs/GC, DOPE/polyTTBA/AuNPs/GC, TBO/polyTTBA/AuNPs/GC, and (d) DOPE + TBO/polyTTBA/AuNPs/GC electrodes in a 0.1 M Britton-Robinson buffer (pH 10.0) + acetonitrile (10 v/v%) containing 1.0 mM mixed PEs at a scan rate of 50.0 mV/s.. (D) LSVs for the DOPE + TBO/polyTTBA/AuNPs/GCE in 0.1 M Britton-Robinson buffer + acetonitrile solution containing 1.0 mM each of DBP, DEHP, DCHP, DEP, and BBP at a scan rate of 50.0 mV/s.

Figure 3. (A) Electropherograms of 1.0 nM DEP, DBP, BBP, DEHP, and DCHP obtained in (a) only FASI and (b) FASS and FASI together at a detection potential of -1.7 V, with a separation field strength of 2.0 kV; injection time of 5.0 s at 2.0 kV; with 10.0 mM Britton-Robinson buffer + acetonitrile (10 v/v%) + 10.0 mM SDS, and low-pH BGE (pH 2.1) (10.0 mM Britton-Robinson buffer + acetonitrile (10 v/v%) + 10.0 mM SDS + 1.0 M urea + AuNPs) as the running buffer. (B) Calibration curves obtained by plotting the signals obtained in Supporting Information, Figure S6. (C) Calibration plot for the (a) DEP and (b) DEHP separation with the SAM under optimized conditions. Inset: Determination of unknown concentrations of PEs in (a) manicure and (b) PVC samples by the SAM. Standard

solutions prepared with the known concentrations of (I) 0.0, (II) 10.0, (III) 25.0, and (IV) 50.0 nM of DEP and DEHP with manicure and PVC, respectively. Experimental conditions same as in Figure 3(A(b)). *The unknown chemical species detected in the real samples are indicated by asterisks.

Figure 4. (A) Microscopic images of Vero non-cancerous mammalian kidney cells in culture (a) without the addition of PEs, inset of (a) Vero cells in higher magnification, (b), (c), and (d) Vero cells with 50.0 nM of PEs after 6, 12, and 24 hrs of incubation, respectively. (e), (f), and (g) Vero cells with 250.0 nM of PEs after 6, 12, and 24 hrs respectively, and (h), (i), and (j) Vero cells with 500.0 nM of PEs after 6, 12, and 24 hrs of incubation, respectively. (B) Electropherograms of 50.0 μ L cell lysate with (a) 50.0, (b) 250.0, and (c) 500.0 nM for (I) 6, (II) 12, and (III) 24 hrs of incubation.



Scheme 1.

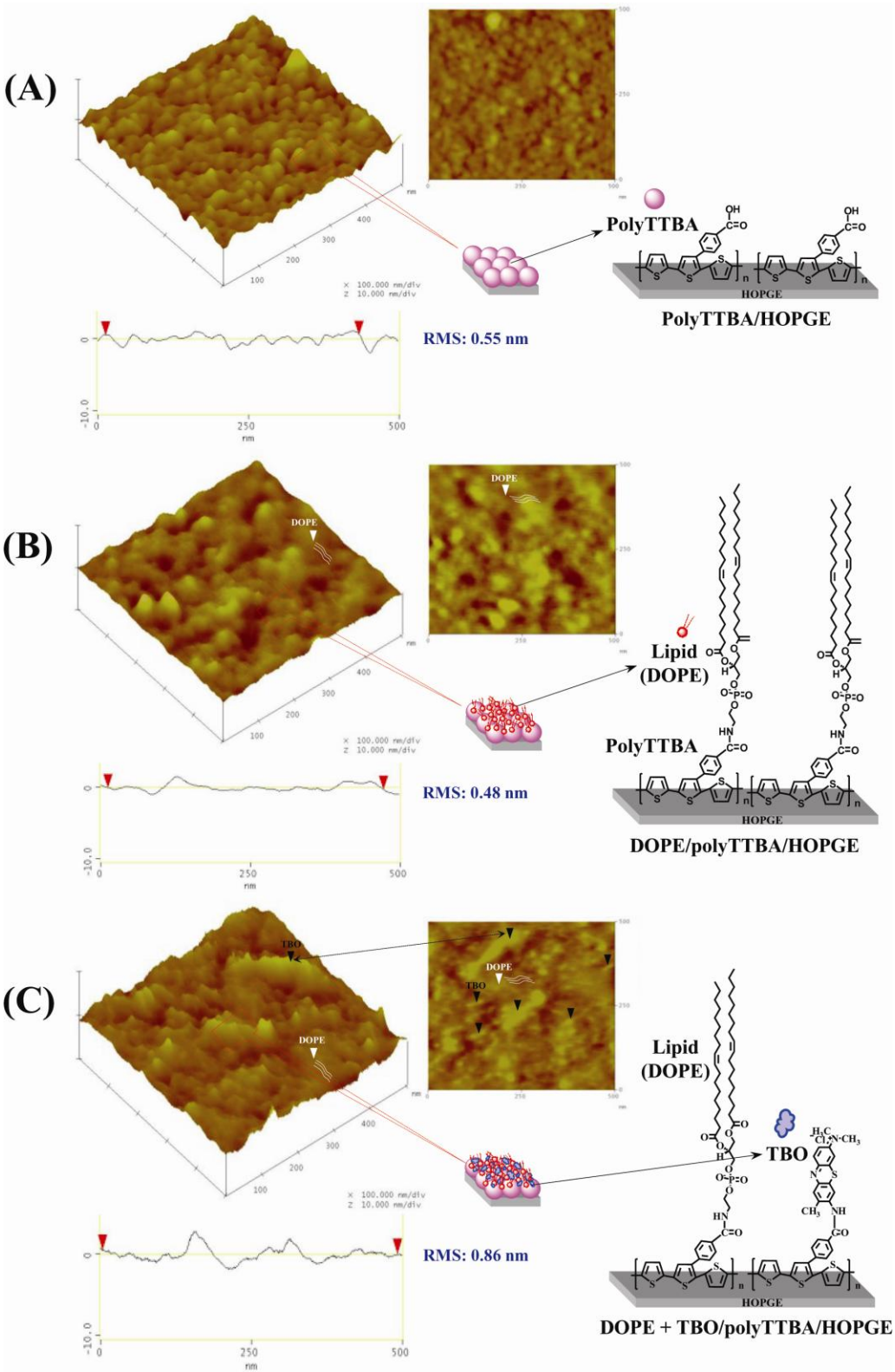


Figure 1.

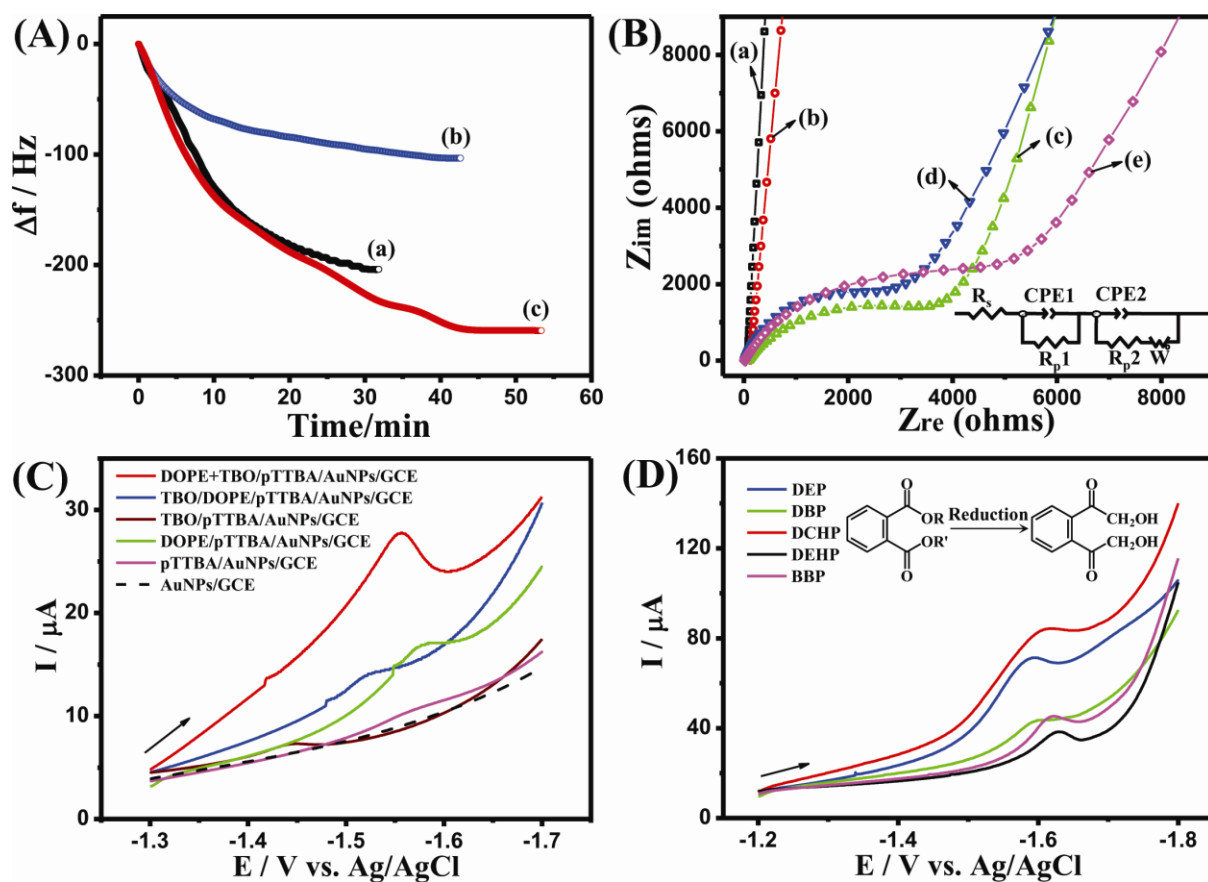


Figure 2.

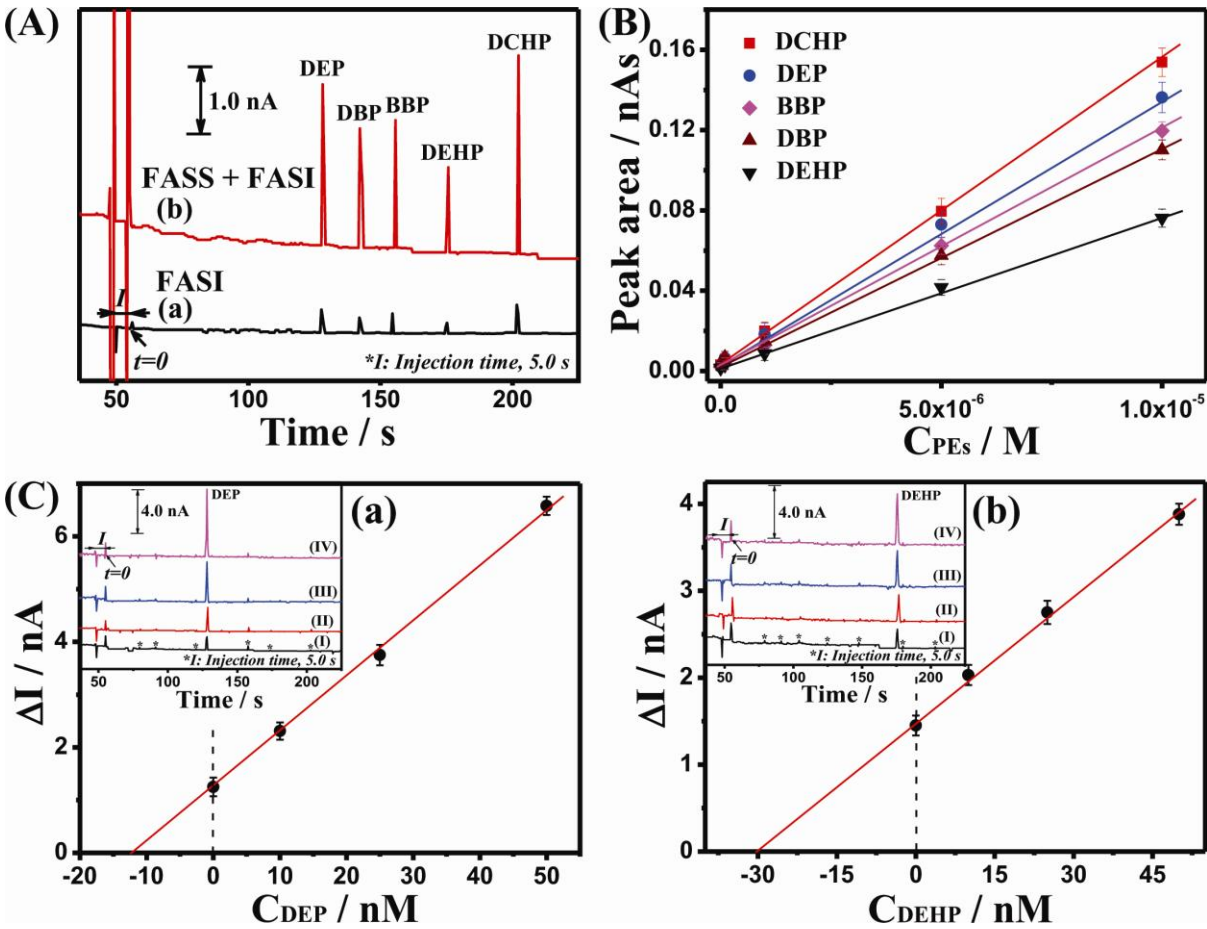


Figure 3.

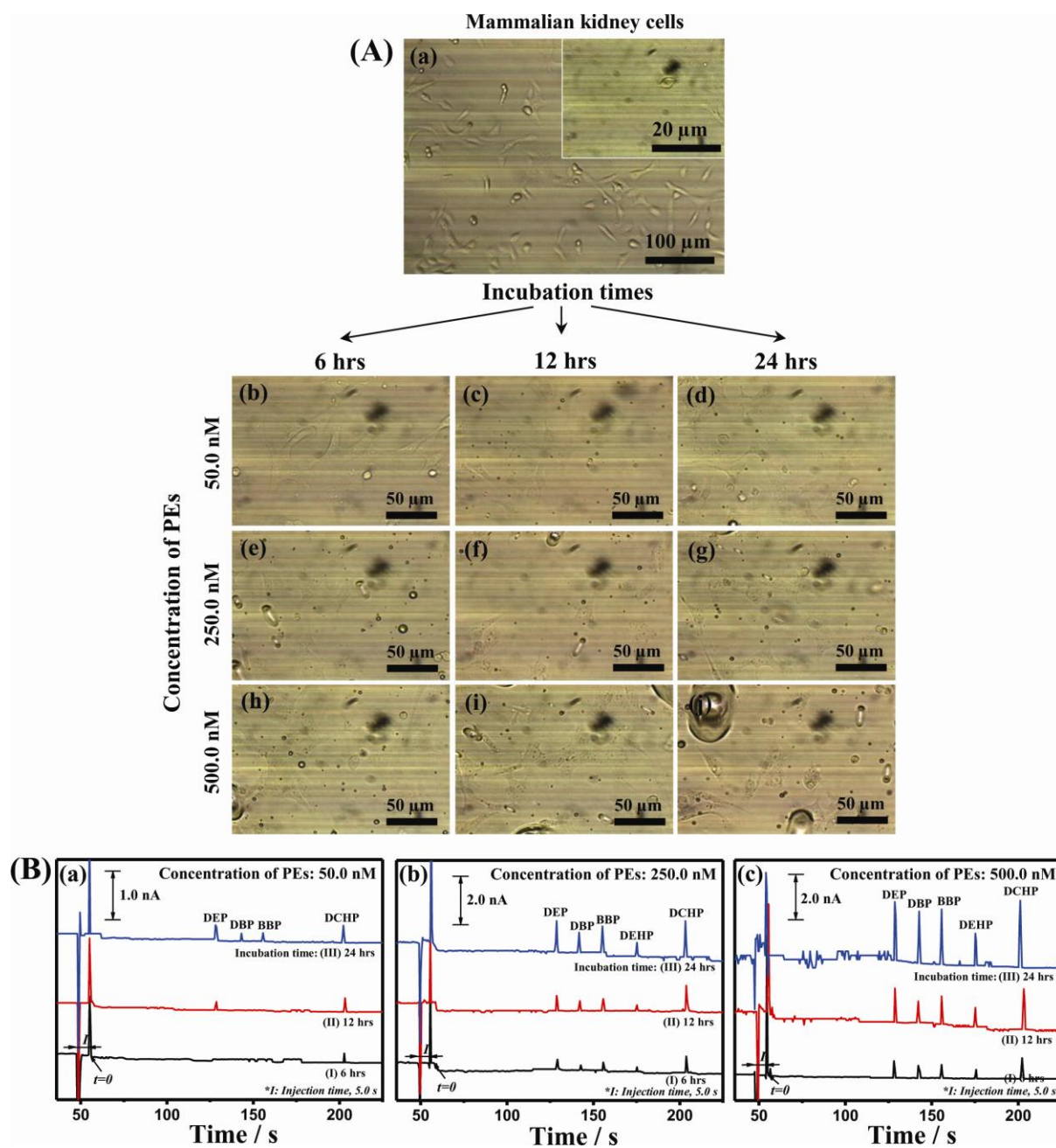


Figure 4.

for TOC only

