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TITLE:

Electrochemical Preparation of Poly(3,4-Ethylenedioxythiophene) Layers on Gold Microelectrodes for Uric Acid-sensing Applications

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KEYWORDS:

Poly(3,4-ethylenedioxythiophene) (PEDOT), cyclic voltammetry, lithium perchlorate, gold microelectrode, propylene carbonate, scanning electron microscopy, flavored milk, electrodeposition

SUMMARY:

We describe aqueous and organic solvent systems for the electropolymerization of poly(3,4-ethylenedioxythiophene) to create thin layers on the surface of gold microelectrodes, which are used for sensing low molecular weight analytes.

ABSTRACT:

Two different methods for the synthesis of poly(3,4-ethylenedioxythiophene) (PEDOT) on gold electrodes are described, using electropolymerization of 3,4-ethylenedioxythiophene (EDOT) monomer in an aqueous and an organic solution. Cyclic voltammetry (CV) was used in the synthesis of PEDOT thin layers. Lithium perchlorate ($LiClO_4$) was used as a dopant in both aqueous (aqueous/acetonitrile (ACN)) and organic (propylene carbonate (PC)) solvent systems. After the PEDOT layer was created in the organic system, the electrode surface was acclimatized by successive cycling in an aqueous solution for use as a sensor for aqueous samples.

The use of an aqueous-based electropolymerization method has the potential benefit of removing the acclimatization step to have a shorter sensor preparation time. Although the aqueous method is more economical and environmentally friendly than the organic solvent method, superior PEDOT formation is obtained in the organic solution. The resulting PEDOT

electrode surfaces were characterized by scanning electron microscopy (SEM), which showed the constant growth of PEDOT during electropolymerization from the organic PC solution, with rapid fractal-type growth on gold (Au) microelectrodes.

INTRODUCTION:

Electrically conducting polymers are organic materials widely used in bioelectronic devices to improve interfaces. Similar to conventional polymers, conducting polymers are easy to synthesize and are flexible during processing[1]. Conducting polymers can be synthesized using chemical and electrochemical methods; however, electrochemical synthesis approaches are particularly favorable. This is mainly due to their ability to form thin films, allow simultaneous doping, capture molecules in the conducting polymer, and most importantly, the simplicity of the synthesis process[1]. In addition, conducting polymers form uniform, fibrous, and bumpy nanostructures, firmly adherent to the electrode surface, which increases the active surface area of the electrode[2].

In the 1980s, certain polyheterocycles, such as polypyrrole, polyaniline, polythiophene, and PEDOT, were developed that showed good conductivity, ease of synthesis, and stability[3,4]. Although polypyrrole is better understood than other polymers (e.g., polythiophene derivatives), it is prone to irreversible oxidation[5]. Thus, PEDOT has certain advantages over the rest as it has a much more stable oxidative state and retains 89% of its conductivity compared to polypyrrole under similar conditions[6]. In addition, PEDOT is known for high electroconductivity (~500 S/cm) and a moderate band gap (i.e., band gaps or energy gaps are regions with no charge and refer to the energy difference between the top of a valence band and the bottom of a conduction band)[7].

Furthermore, PEDOT has electrochemical properties, needs lower potentials to be oxidized, and is more stable over time than polypyrrole after being synthesized[7]. It also has good optical transparency, which means its optical absorption coefficient, especially in the form of PEDOT-polystyrene sulfonate (PEDOT-PSS), is in the visible region of the electromagnetic spectrum at 400–700 nm[7]. In the formation of PEDOT electrochemically, EDOT monomers oxidize at the working electrode to form radical cations, which react with other radical cations or monomers to create PEDOT chains that deposit on the electrode surface[1].

Different controlling factors are involved in the electrochemical formation of PEDOT films, such as electrolyte, electrolyte type, electrode setup, deposition time, dopant type, and solvent temperature[1] PEDOT can be generated electrochemically by passing current through an appropriate electrolyte solution. Different electrolytes such as aqueous (e.g., PEDOT-PSS), organic (e.g., PC, acetonitrile), and ionic liquids (e.g., 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIMBF_4$)) can be used [8].

One of the advantages of PEDOT coatings is that it can significantly decrease the impedance of a Au electrode in the 1 kHz frequency range by two or three orders of magnitude, which makes it helpful to increase the sensitivity of direct electrochemical detection of neural activity[9]. Moreover, the charge storage capacity of the PEDOT-modified electrodes increases and results in faster and lower potential responses when stimulation charge is transferred through PEDOT[10].

In addition, when polystyrene sulfonate (PSS) is used as a dopant for PEDOT formation on Au microelectrode arrays, it creates a rough, porous surface with a high active surface area, lower interface impedance, and higher charge injection capacity[11]. For the electropolymerization step, EDOT-PSS usually makes a dispersion in an aqueous electrolyte.

However, EDOT is soluble in chloroform, acetone, ACN, and other organic solvents such as PC. Therefore, in this study, a mixture of water was used with a small volume of ACN in a 10:1 ratio to make a soluble EDOT solution before electropolymerization starts. The purpose of using this aqueous electrolyte is to omit the acclimatization step in the preparation of PEDOT-modified microelectrode and shorten the steps. The other organic electrolyte used to compare with the aqueous/ACN electrolyte is PC. Both electrolytes contain LiClO4 as a dopant to help in oxidizing the EDOT monomer and forming the PEDOT polymer.

Microelectrodes are voltammetric working electrodes with smaller diameters than macroelectrodes, approximately tens of micrometers or less in dimension. Their advantages over macroelectrodes include enhanced mass transport from the solution toward the electrode surface, generating a steady-state signal, a lower ohmic potential drop, a lower double-layer capacitance, and an increased signal-to-noise ratio[12]. Similar to all solid electrodes, microelectrodes need to be conditioned before analysis. The appropriate pretreatment or activation technique is mechanical polishing to obtain a smooth surface, followed by an electrochemical or chemical conditioning step, such as potential cycling over a particular range in a suitable electrolyte[13].

CV is very commonly used in the electrochemical polymerization of PEDOT by inserting electrodes in a monomer solution that involves a suitable solvent and dopant electrolyte. This electrochemical technique is beneficial in providing direction information such as the reversibility of conducting polymer doping processes and the number of transferred electrons, diffusion coefficients of analytes, and the formation of reaction products. This paper describes how two different electrolytes used for the electropolymerization of PEDOT can generate thin nanostructure films with a potential sensing application that depends on the morphology and other intrinsic properties.

PROTOCOL:

1. Preparing analytical solutions

1.1. Preparing 0.1 M EDOT in an organic solution

1.1.1. Weigh out 0.213 g of $LiClO_4$ and transfer it into a 20 mL volumetric flask.

1.1.2. Use a measuring cylinder to take 20 mL of PC from the bottle.

1.1.3. Add PC to the 20 mL volumetric flask containing $LiClO_4$. Mix the solution by placing the flask in an ultrasonic bath for 30 min. Transfer the solution to a 20 mL glass vial.

1.1.4. Cover the vial with aluminum foil and insert a long needle attached to a nitrogen pipe into the solution to degas for 10 min. Then, remove the aluminum foil and cap the vial tightly.

NOTE: Prepare $LiClO_4$ fresh on the day of the experiment.

1.1.5. Before the electrochemical test, transfer 1 mL of the prepared $LiClO_4$ solution (0.1 M) into an electrochemical cell (see the **Table of Materials**).

1.1.6. Use a micropipette (10–100 µL) to add 10.68 µL of EDOT monomer (density: 1.331 g/mL) to the electrochemical cell containing the prepared $LiClO_4$ solution.

1.1.7. Run the CV method (see section 3.4 for CV parameters) to start electropolymerization of EDOT on the bare Au microelectrode surface after inserting all electrode setups in the solution. Use this modified electrode to characterize the surface by scanning electron microscopy (SEM).

1.1.8. To use this modified electrode for sensing purposes, first acclimatize its surface to an aqueous solution by running CV scans in the sodium perchlorate ($NaClO_4$) solution (see section 3.4 for CV parameters).

1.1.9. Use this organically PEDOT-modified and acclimatized microelectrode (from 1.1.8) to run CV (see section 3.4 for its CV parameters) of a phosphate buffer solution to be used as a background scan.

NOTE: Rinse the electrode after each step.

1.1.10. Finally, take out the electrode from the buffer solution without rinsing, and immediately insert it into uric acid solutions or milk samples for running CV scans (see section 3.4 for CV parameters).

1.2. Preparing 0.01 M EDOT in an aqueous solution

1.2.1. Use a micropipette to take 10.68 µL of EDOT and add to 1 mL of ACN in a glass vial.

1.2.2. Add 9 mL of deionized water (18.2 MΩ/cm at 25 °C) to the vial to prepare 10 mL of 0.01 M EDOT solution.

1.2.3. Add 0.11 g of $LiClO_4$ powder to the prepared EDOT solution to obtain 0.1 M $LiClO_4$ solution, and mix gently.

NOTE: Prepare the electrolyte solutions freshly on the day of the experiment.

1.2.4. Transfer the prepared solution to the electrochemical cell and start electropolymerization of 0.01 M EDOT on the electrode surface by the CV method (see section 3.4 for CV parameters)

after inserting the electrode in the aqueous/ACN solution.

1.2.5. Characterize the surface of this modified electrode by SEM.

1.3. Preparing 0.1 M sodium perchlorate solution

1.3.1. Weigh out 0.245 g of $NaClO_4$ and transfer it to a glass vial containing 20 mL of deionized water (18.2 MΩ/cm at 25 °C).

1.3.2. Use this solution to acclimatize the surface of the organically made PEDOT-modified Au microelectrode to an aqueous solution and to remove excess EDOT. For this purpose, rinse the electrode and insert it into the $NaClO_4$ solution; then run CV for 10 cycles (see section 3.4 for CV parameters).

1.4. Preparing buffer solution

1.4.1. Weigh out 13.8 g of sodium dihydrogen phosphate (NaH_2PO_4. $1H_2O$) in a weighing boat. Transfer it to a 500 mL volumetric flask (i.e., the required final volume) and top it up to the line with deionized water (18.2 MΩ/cm at 25 °C).

1.4.2. Place the flask in an ultrasonic bath until the powder dissolves completely in the water, resulting in a 0.2 M solution.

1.4.3. In a new weighing boat, weigh out 17.8 g of disodium hydrogen phosphate (Na_2HPO_4. $2H_2O$) and transfer it to another 500 mL volumetric flask. Top it up with deionized water to obtain a 0.2 M solution. Place the flask in an ultrasonic bath to dissolve properly.

1.4.4. Mix 62.5 mL of sodium dihydrogen phosphate solution with 37.5 mL of disodium hydrogen phosphate solution in a measuring cylinder and transfer the mixture to a 250 mL glass bottle (see the **Table of Materials**). Top it up with another 100 mL of deionized water to obtain 200 mL of 0.1 M of phosphate buffer solution, pH 6.6. Refrigerate the phosphate buffer for long-term use.

NOTE: Bring the buffer to room temperature before each experiment.

1.5. Preparing target analyte solutions

1.5.1. Weigh out 0.0084 g of uric acid (UA) in a weighing boat, and dissolve it in 50 mL of phosphate buffer (pH 6.6) in a volumetric flask to obtain a 1 mM UA solution.

1.5.2. Degas the solution by nitrogen purging for 10 min.

NOTE: It is advisable to prepare the UA solution fresh on the day of the experiment.

1.6. Preparing milk samples for analysis

1.6.1. Obtain a whole milk sample and some milk samples with different flavors (e.g., Espresso milk, Caramel/white chocolate milk, and Belgian chocolate milk) from a local supermarket for electroanalysis. Do not pretreat or dilute the milk samples.

1.6.2. Use a 5 mL micropipette to take 5 mL of each milk sample from the freshly opened bottles.

1.6.3. First, run CV of phosphate buffer, pH 6.6, as a background signal. Then, add the 5 mL milk sample into the electrochemical cell, and insert freshly and organically made, PEDOT-modified Au microelectrode and other electrodes into the milk samples and run CV. See section 4 of the protocol for how to analyze the collected data.

1.7. Preparing electrode pretreatment solutions

1.7.1. Weigh out 0.2 g of sodium hydroxide (NaOH) powder and transfer it to a 50 mL volumetric flask to prepare a 0.1 M solution.

1.7.2. Use the 0.1 M NaOH solution to remove the residue of PEDOT formed on the microelectrode surface after each run.

1.7.3. Use a glass pipette to withdraw 27.2 mL from a 98% sulfuric acid (H_2SO_4) bottle. Add it very slowly to a 1 L volumetric flask half-filled with deionized water.

1.7.4. Top up the flask to the line with deionized water to prepare 1 L of a 0.5 M H_2SO_4 solution.

NOTE: Prepare H_2SO_4 solution under a fume hood for safety. Use the H_2SO_4 solution in the final electrochemical cleaning step of the microelectrode.

2. Pretreatment of the gold microelectrode

2.1. Polish the Au microelectrode (10 µm diameter, 3.5 mm width x 7 cm long) on an alumina polishing pad placed on a glass polishing plate (dimensions: 3" x 3" squares) using an alumina slurry for 30 s with circular and eight-shaped hand motions during polishing.

2.2. Rinse the Au microelectrode with deionized water, insert it in a glass vial containing 15 mL of absolute ethanol (LR grade), and ultrasonicate for 2 min.

2.3. Rinse the Au microelectrode with ethanol and water and again ultrasonicate it for 4 min in deionized water to remove excess alumina from the electrode surface.

2.4. Finally, remove additional impurities by cycling in 0.5 M H_2SO_4 for 20 segments between -0.4 and 1.6 V potentials (vs. Ag/AgCl) at a 50 mV/s scan rate. Ensure there are two clear peaks due to the formation and reduction of gold oxide at consistent anodic and cathodic potentials each time the electrode is cleaned in H_2SO_4.

3. Cyclic voltammetry technique

3.1. Use a suitable potentiostat to run CV as the electrochemical technique of interest.

3.2. Turn on the potentiostat and the computer attached to it. Make sure the system is connected.

3.3. To test the communication between the computer and the instrument, start the software and switch on the instrument. Use the **Hardware Test** command under the **Setup** menu. If a **Link Failed** error appears, check the connection and port settings.

3.4. Open the potentiostat software on the computer, and in the **method** command, choose the **cyclic voltammetry method**. Then, click on **parameters**, and enter the experimental parameters.

3.4.1. Use the following CV parameters to run PEDOT electropolymerization in an organic electrolyte on the bare Au microelectrode: initial potential: -0.3 V, final potential: -0.3 V, high potential: 1.2 V, number of segments: 8, scan rates: 100 mV/s, direction: positive.

3.4.2. Use the following CV parameters to run PEDOT electropolymerization in an aqueous/ACN electrolyte on the bare Au microelectrode: initial potential: -0.3 V, final potential: -0.3 V, high potential: 1.2 V, number of segments: 20, scan rates: 100 mV/s, direction: positive.

3.4.3. Use the following CV parameters to run the acclimatization step of the organically made PEDOT-modified Au microelectrode: initial potential: -0.2 V, final potential: -0.2 V, high potential: 0.8 V, number of segments: 20, scan rates: 100 mV/s, direction: positive.

3.4.4. Use the following CV parameters for UA standard solutions and phosphate buffer (pH 6.6) with the bare Au microelectrode: initial potential: 0 V, final potential: 0 V, high potential: 1 V, number of segments: 2, scan rates: 100 mV/s, and direction: positive.

3.4.5. Use the following CV parameters for UA standard solutions and phosphate buffer (pH 6.6) on the organically made, PEDOT-modified Au microelectrode: initial potential: 0 V, final potential: 0 V, high potential: 0.6 V, number of segments: 2, scan rates: 100 mV/s, and direction: positive.

3.4.6. Use the following CV parameters for the milk samples and phosphate buffer (pH 6.6) on the organically made, PEDOT-modified Au microelectrode: initial potential: 0 V, final potential: 0 V, high potential: 0.8 V, number of segments: 2, scan rates: 100 mV/s, direction: positive.

3.5. Prepare three electrode setups in a glass electrochemical cell including a working electrode (Au microelectrode (10 µm diameter)), a reference electrode (e.g., silver/silver chloride (Ag/AgCl) in 3 M sodium chloride (NaCl), and a platinum wire counter electrode.

3.6. Pass these clean and dried electrodes through the holes of an electrode holder attached to

a stand. Then, drag the holder above the electrochemical cell to insert the electrodes in the target solution or sample.

3.7. Ensure that there are no bubbles on the electrode surfaces.

3.7.1. If there are bubbles, remove the electrodes, rinse with deionized water again and pat dry with a tissue. Place the electrodes back into the stand holder and in the solution.

3.7.2. If there are bubbles around the reference electrode, tap the tip gently.

3.7.3. If there are bubbles around the counter electrode after it starts running, clean the counter electrode. If the CV scan becomes noisy, clean the electrode surface and check the system connections, wires, and clips.

3.8. Ensure that all the three wire connections for reference, working, and counter electrodes are correctly connected, and then start the experiment by clicking on **Run** at the bottom.

3.9. Run all experiments at room temperature. For milk samples, let the temperature of the milk samples reach the ambient temperature before running CV.

4. Data collection and analysis

4.1. After running CV, save the data in the desired format (CSV or Bin) in a folder, and then use a USB memory stick to collect it. Analyze the data using appropriate software. Convert CSV files to spreadsheets for easier analysis.

NOTE: If data are saved in the format of a Binary file, convert it to the format of **Text Comma** before data collection in a USB memory stick.

4.2. To analyze the CV of milk samples, subtract the CV of milk from the background CV (i.e., CV of phosphate buffer (pH 6.6) taken before running each milk sample) to produce curves due to milk profile oxidation.

5. Techniques to characterize PEDOT

5.1. Use a specific type of high-performance SEM to characterize the PEDOT layers made in different electrolytes.

NOTE: Here, FEI Quanta 200 ESEM FEG was used; it is equipped with a Schottky field emission gun (FEG) for better spatial resolution. This instrument provides different working modes such as high vacuum, low vacuum, and environmental SEM modes and is equipped with a SiLi (Lithium drifted) Super Ultra-Thin Window EDS detector.

5.2. Check the surface morphology of both bare and PEDOT-modified Au (PEDOT-Au)

microelectrodes by SEM after PEDOT electropolymerization in organic and aqueous solutions. Perform the PEDOT electropolymerization on bare Au microelectrodes in aqueous/ACN and organic solutions immediately before checking them by SEM.

5.3. Place the freshly prepared electrodes (a bare Au microelectrode and two of the PEDOT-Au microelectrodes) on the SEM stage horizontally, with their head above the stage at a certain angle.

REPRESENTATIVE RESULTS:

Cyclic voltammetry is an easy technique to form a thin PEDOT layer on a Au microelectrode surface to increase the electrode conductivity and sensitivity during electrochemical sensing of target analytes. This protocol demonstrates the method of electropolymerization of 0.1 M EDOT from an organic solution compared to 0.01 M EDOT from an aqueous electrolyte solution. Running 10 cycles in aqueous/ACN solution results in a moderate growth of PEDOT comparable to that observed with the 4 cycles in $LiClO_4$/PC solution. **Figure 1** shows a distinct difference between EDOT electropolymerized in aqueous/ACN and organic solutions, with the subsequent PEDOT layers formed by applying CV. It is evident that upon cycling from -0.3 to +1.2 V (vs. Ag/AgCl in 3 M NaCl) at a 100 mV/s scan rate, the polymer started to oxidize at 0.9 V in both electrolyte solutions (**Figure 1A** and **Figure 1C**), with an oxidation peak seen at 1 V in the aqueous/ACN solution.

Upon closer inspection, the PEDOT layers made in the organic solution after 4 cycles display higher current values (~2.9 µA) at 1.2 V compared with the current value (0.23 µA) seen for PEDOT layers formed at this potential in the aqueous/ACN solution. When the number of electropolymerization cycles increases during CV runs, the new layers of PEDOT are made gradually on the electrode surface to increase the thickness of layers. This could be due to the redox reactions occurring in the internal PEDOT between the potential range of 0 to 0.7 V (**Figure 1B** and **Figure 1D**)**. Figure 1B** and **Figure 1D** depict the narrowing down of the potential range in a spreadsheet to interpret the PEDOT growth correctly. The current density values on the right side of each graph were calculated by dividing the current values on the left side of the graph by the geometric surface area of the unmodified Au microelectrode (78.5×10^{-8} cm^2, $r = 5 \times 10^{-4}$ cm).

SEM analysis was performed to confirm the efficiency of PEDOT layer formation by electropolymerization in the two electrolyte solutions (**Figure 2A–F**)**.** The images taken by SEM were chosen at different magnifications (4000x, 30000x, and 60000x). The geometric surface area of bare and PEDOT-Au microelectrodes can be established using these images. **Figure 2A** confirms a diameter of ~10 µm for the bare gold microelectrode; hence, the surface area is calculated to be ~78.5×10^{-8} cm^2. The diameter of the PEDOT nanostructure formed in the organic solution after 4 cycles at the surface of the Au microelectrode was ~40 µm (**Figure 2C,D**). By contrast, the PEDOT growth on the electrode surface was lower after 10 cycles of electropolymerization. It is seen as mountainous polymeric features on the electrode circumference with a depression in the center (**Figure 2E,F**).

The SEM images provide evidence for the superiority of the PEDOT growth in the organic solution

compared to the aqueous/ACN system and the creation of a very porous nanostructure extending out from the microelectrode in a cauliflower-like shape. This PEDOT microelectrode prepared in an organic solution was used for sensing applications, particularly for UA detection in standard solutions and milk samples. **Figure 3** shows the CV for the detection of UA in a standard solution at a bare Au microelectrode and the PEDOT sensor. The performance of the bare Au microelectrode for UA detection is characterized by steady-state currents obtained at potentials higher than 0.8 V due to radial diffusion of UA to the electrode surface (**Figure 3A**). A linear calibration curve was plotted based on the average currents at 0.8 V for the UA concentration range of 62.5 to 1000 µM after three replicate CV runs (**Figure 3B**).

By comparing the slope of the calibration curve equations, the PEDOT microelectrode was found to have 100 times higher sensitivity than the bare microelectrode. Interestingly, the detected UA range using the PEDOT sensor made in an organic solution was lower, from 6.25 to 200 µM, calculated by measuring the current value at the tip of the sharp anodic peak **(Figure 3C,D**). The calibration curve data for the PEDOT electrode were used to measure the limit of detection (LOD) and limit of quantification (LOQ) of the UA for the modified electrode. The slope of the calibration curve equation (*b*) and the evaluated standard error of the intercept (*s*) were used to measure the LOD and LOQ values (95% confidence level)—7 µM and 24 µM[14], respectively—by using equations (**1**) and (**2**).

$$LOD = 3s/b \quad (\mathbf{1})$$

$$LOQ = 10s/b \quad (\mathbf{2})$$

The sensitivity of the organically made PEDOT-modified sensor is an important factor. This is calculated by dividing the calibration curve slope by the geometric surface area of the working electrode, which is 397 µA μM^{-1} cm^{-2}.

Another application of the PEDOT sensor synthesized in the organic solution was to analyze UA content in real samples, e.g., regular fresh milk and selected flavored milk samples (**Figure 4)**. The advantage of this technique is that UA levels in milk samples can be measured without any pretreatment or dilution using CV. The performance of this PEDOT-Au microelectrode sensor was compared to the PEDOT-modified glassy carbon macroelectrode (PEDOT-GC) prepared by the same method in the organic solution[15]. The anodic peak current for UA in regular milk at 0.35 V (vs. Ag/AgCl) using the PEDOT microelectrode was ~28.4 nA, which is equivalent to 82.7 µM using the equation of the calibration curve in **Figure 3D** ($y = 0.3x + 2.6$, $R^2 = 0.993$). This value was ~83.4 µM for UA in the regular milk determined using the PEDOT-GC[15]. The second large oxidation peak in the CV scan of regular milk at 0.65 V (**Figure 4A**) is related to oxidizable compounds, including electroactive amino acids such as cysteine, tryptophan, and tyrosine[15,16]. The current density of this peak from the regular milk is over 200 times larger than that obtained using a previously reported PEDOT-GC[15]. This shows a more sensitive response of the microelectrode covered by PEDOT layers compared to the PEDOT-modified macroelectrode.

The CV scans obtained for caramel and white chocolate milk samples can be seen in **Figure 4A.** It shows a clear peak at 0.36 V for UA, along with an additional peak current of ~42 nA at 0.56 V

that is merged with the peak at 0.66 V. This additional peak at 0.56 V can be related to the presence of vanillic acid, one of the ingredients of flavored milk. The CV of the Belgian chocolate milk sample indicates a new set of anodic peaks at 0.26 V, 0.36 V, and 0.66 V and a cathodic peak at 0.22 V. The chocolate profile resembles the catechin redox profile along with the other polyphenolic antioxidants present in chocolate or cocoa[15]. Thus, the catechin oxidation and reduction peaks appear at 0.26 V and 0.22 V, respectively. The 0.36 V peak current, which appears as a sharp peak at the tail of the catechin peak, is due to UA oxidation. **Figure 4B** shows a CV of Colombian espresso milk sample, which exhibits broad anodic and cathodic peak currents at 0.35 V and 0.23 V, respectively, at the PEDOT-Au, which are due to the major phenolic antioxidants in coffee, namely, chlorogenic and caffeic acids. Because the geometric surface area of the PEDOT microelectrode is higher than that of the PEDOT macroelectrode, the current densities of the UA peaks in these milk samples are ~150 to 500 times larger at the PEDOT-Au[15].

[Place Figure 1 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Electropolymerization of PEDOT on a gold microelectrode. PEDOT prepared by (**A, B**) 10 CV scans in an aqueous solution (0.01 M EDOT in 1 mL ACN + 9 mL deionized water + 0.1 M $LiClO_4$); and (**C, D**) using 4 CV scans in an organic electrolyte solution (0.1 M EDOT in 1 mL of 0.1 M $LiClO_4$/PC). **B** and **D** are expanded versions of **A** and **C** to visualize the PEDOT currents clearly. Scan rate = 100 mV/s. This figure has been modified from [15]. Abbreviations: PEDOT = poly(3,4-ethylenedioxythiophene); CV = cyclic voltammetry; EDOT = 3,4-ethylenedioxythiophene; ACN = acetonitrile; $LiClO_4$ = lithium perchlorate; Ag = silver; AgCl = silver chloride.

[Place Figure 2 here]

Figure 2: SEM images. (**A** and **B**) Bare gold microelectrode (Au). PEDOT-modified gold microelectrodes prepared in (**C** and **D**) organic solution after 4 cycles of electropolymerization and (**E** and **F**) aqueous solution after 10 cycles of electropolymerization at different magnifications. This figure has been modified from[15]. Abbreviations: SEM = scanning electron microscopy; PEDOT = poly(3,4-ethylenedioxythiophene).

[Place Figure 3 here]

Figure 3: Cyclic voltammograms for different concentrations of UA in phosphate buffer, pH 6.6. (**A**) Bare gold microelectrode (background subtracted) and (**C**) PEDOT-modified gold microelectrode (background subtracted), measurements taken immediately after inserting the electrode into the solution at a scan rate of 100 mV/s. (**B**) Plot of limiting current at 0.8 V versus UA concentration on the bare gold microelectrode. (**D**) Plot of anodic peak current ($I_{p.a}$/μA) versus UA concentration on the PEDOT-modified gold microelectrode. (n=3). This figure has been modified from[15]. Abbreviations: UA = uric acid; PEDOT = poly(3,4-ethylenedioxythiophene).

[Place Figure 4 here]

Figure 4: Cyclic voltammograms (background subtracted). (**A**) Regular milk, Belgian chocolate milk, caramel, and white chocolate milk, and (**B**) regular milk and Colombian espresso milk on a PEDOT-modified gold microelectrode (10 µm diameter) at 100 mV/s. This figure has been modified from[15]. Abbreviation: PEDOT = poly(3,4-ethylenedioxythiophene).

DISCUSSION:

The CV method allows for fast and simple measurement of different analytes in foods, wine and beverages, plant extracts, and even biological samples. This technique produces a wide variety of data, including oxidation/reduction peak potentials, peak current values of the target analyte (proportional to concentration), and all other current and potential values after each CV run. Although using CV is relatively easy, the collected data sometimes need to be converted from Binary files to Text Comma format, depending on the potentiostat system used. For instance, in the case of the CH instrument, the data can be saved in Text Comma or CSV formats directly after each run. This makes the data analysis easier in a spreadsheet after converting texts to columns. After the CV scans of the milk or UA standard samples were obtained at the same potential ranges, they were plotted on a single graph for direct comparison. To present the data for publications, graphs can also be plotted in Origin SigmaPlot and then exported as TIF or the required graphic file types.

Common problems with this method can be artifacts in the CV trace. These can arise from electrical connection errors, likely due to the connection clips (i.e., clips that attach wires to each electrode) that have become rusted or due to gold microelectrodes not being cleaned properly. Using sandpaper to remove rust from the clips or replacing them, and re-cleaning the microelectrode and re-running CV cycles after inserting it in the H_2SO_4 solution may resolve the issue.

Cleaning the microelectrode is an important step in this experiment, which can otherwise result in a low current signal or noise. Cleaning the microelectrode is also very important as bubbles can form when the microelectrode is not very clean. When the locations of the gold oxidation and reduction peaks and the peak heights obtained are consistent and correct, the electrode is ready to run the electropolymerization. When the potentiostat or electrode connections are faulty, there will be noise in the CV scan, or the output will appear like spreading dots. Before a run, it is important to double-check that all the electrode connections are connected correctly, that there is no gas bubble near the tip of the Ag/AgCl reference electrode, and that the electrodes are not touching in the electrochemical cell. The replacement of the clips and connection wires or tapping the reference electrode tip with a finger can be a useful troubleshooting approach.

During the formation of a PEDOT electrode, as the chosen conducting polymer, the organic electrolyte ($_{LiClO4}$ in PC) and the aqueous $NaClO_4$ solutions should be degassed before running the electropolymerization. It is imperative to use an EDOT chemical that has not expired or oxidized or been contaminated by other analytical grade chemicals. The fresh PEDOT layers that are formed every time on the electrode surface are different in terms of current growth. If the procedure is kept constant and the electrode is cleaned sufficiently, the CV cycles of

electropolymerization would grow by the same current value each time, confirming the accuracy and consistency of the method. It is also worth noting that the amount of the EDOT monomer used in the organic solution was 10 times higher than the EDOT monomer in the aqueous/ACN solution. Although this may seem not comparable, it was considered preferable because our preliminary experiments showed that an aqueous 0.1 M EDOT solution did not form a stable PEDOT layer due to lower solubility in an aqueous electrolyte solution. In contrast, the PEDOT layer formed using 0.01 M EDOT in an organic solution did not have sufficient growth on the electrode surface compared to the aqueous 0.1 M EDOT solution. Hence, those EDOT amounts used for organic and aqueous electropolymerization were selected for this study.

One of the limitations of the CV method when bare electrodes are used is the difficulty to separate peaks when interfering agents exist. However, this problem was resolved when PEDOT was used to modify the electrode surface. For instance, when UA was the target analyte to be detected in milk, it was identified separately from its interfering agent, ascorbic acid, due to the redox mediating role of PEDOT, leading to an earlier and well-separated peak for ascorbic acid. At the same time, even with the PEDOT electrode, when analyzing flavored milk, it can be challenging to separate the UA peak properly from the other ingredients that have close oxidation potentials to UA, leading to a merging of peaks.

To conclude, although troubleshooting may be required intermittently, the use of the CV and PEDOT nanolayers on the electrode surface is advantageous for detecting target analytes such as UA in standard solutions and complex matrix solutions, such as milk samples, without any pretreatments. Compared to the high-performance liquid chromatography technique, this CV method is fast and does not need time-consuming pretreatment steps to remove fat or proteins from milk samples. Further, PEDOT makes the microelectrode highly selective and sensitive, giving a sharp peak for UA analysis.

ACKNOWLEDGMENTS:

Thanks to the funding provided by the New Zealand Ministry of Business, Innovation and Employment (MBIE) within the “High Performance Sensors” program.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

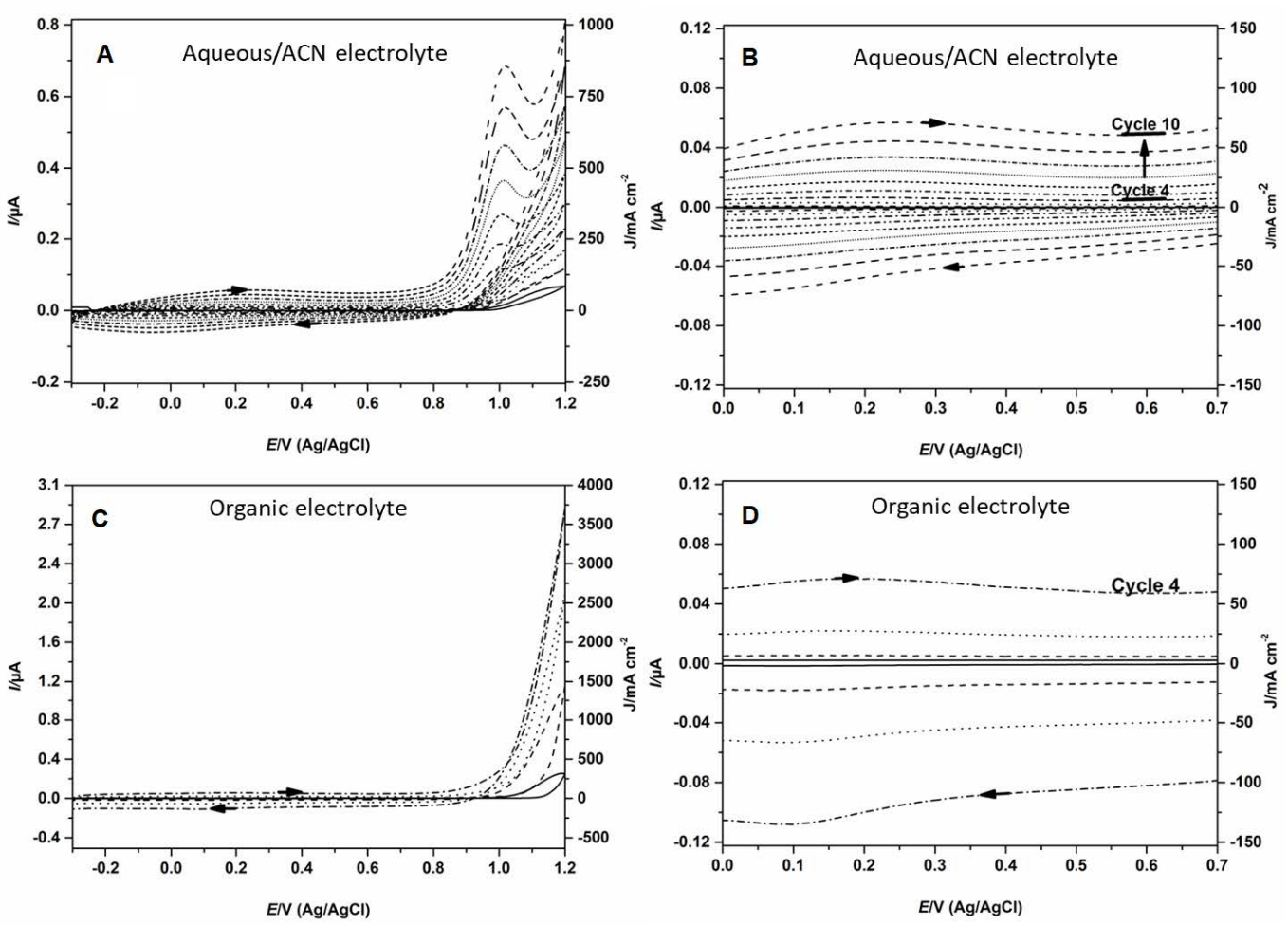

Figure 2

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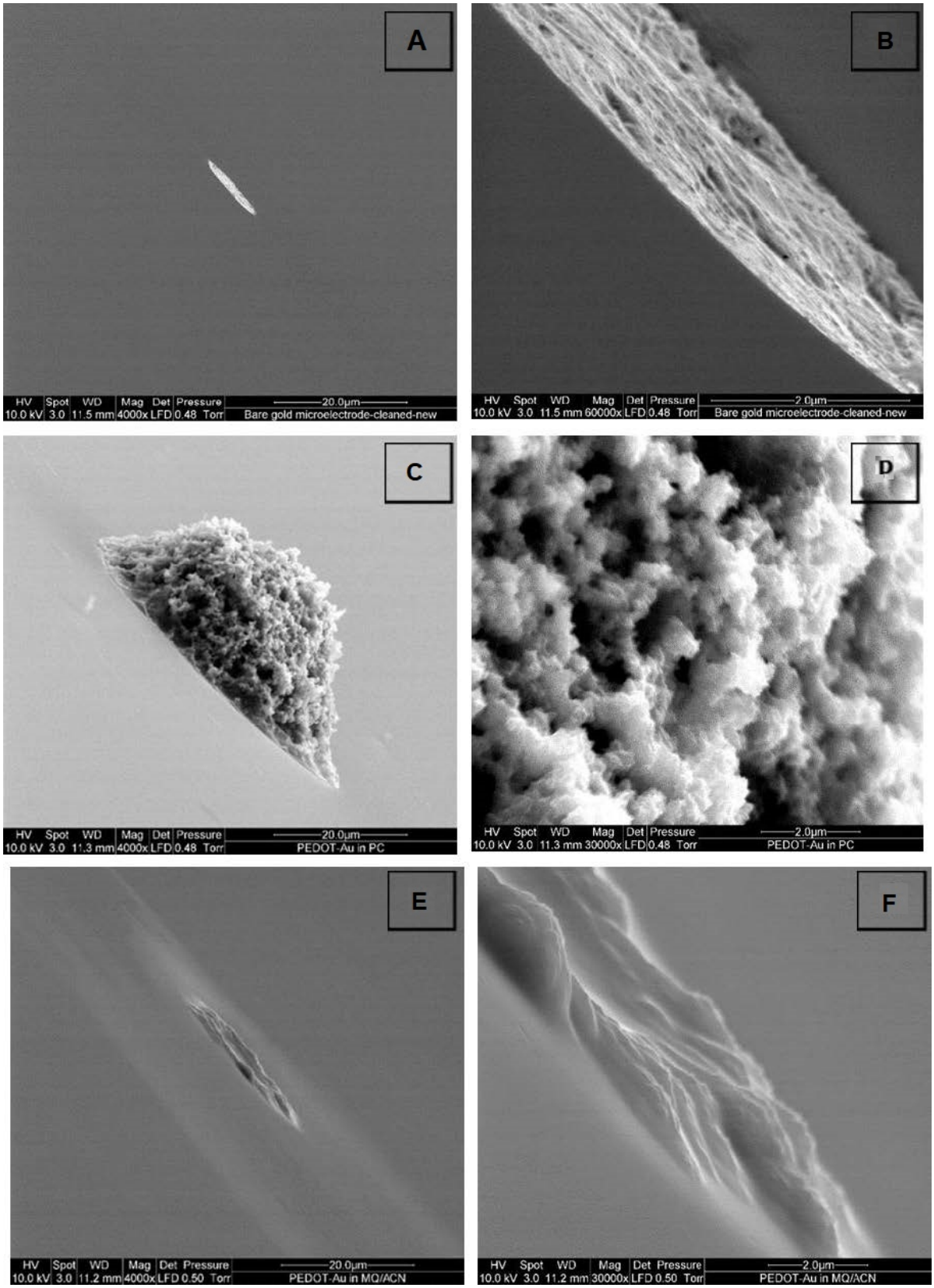


Figure 3

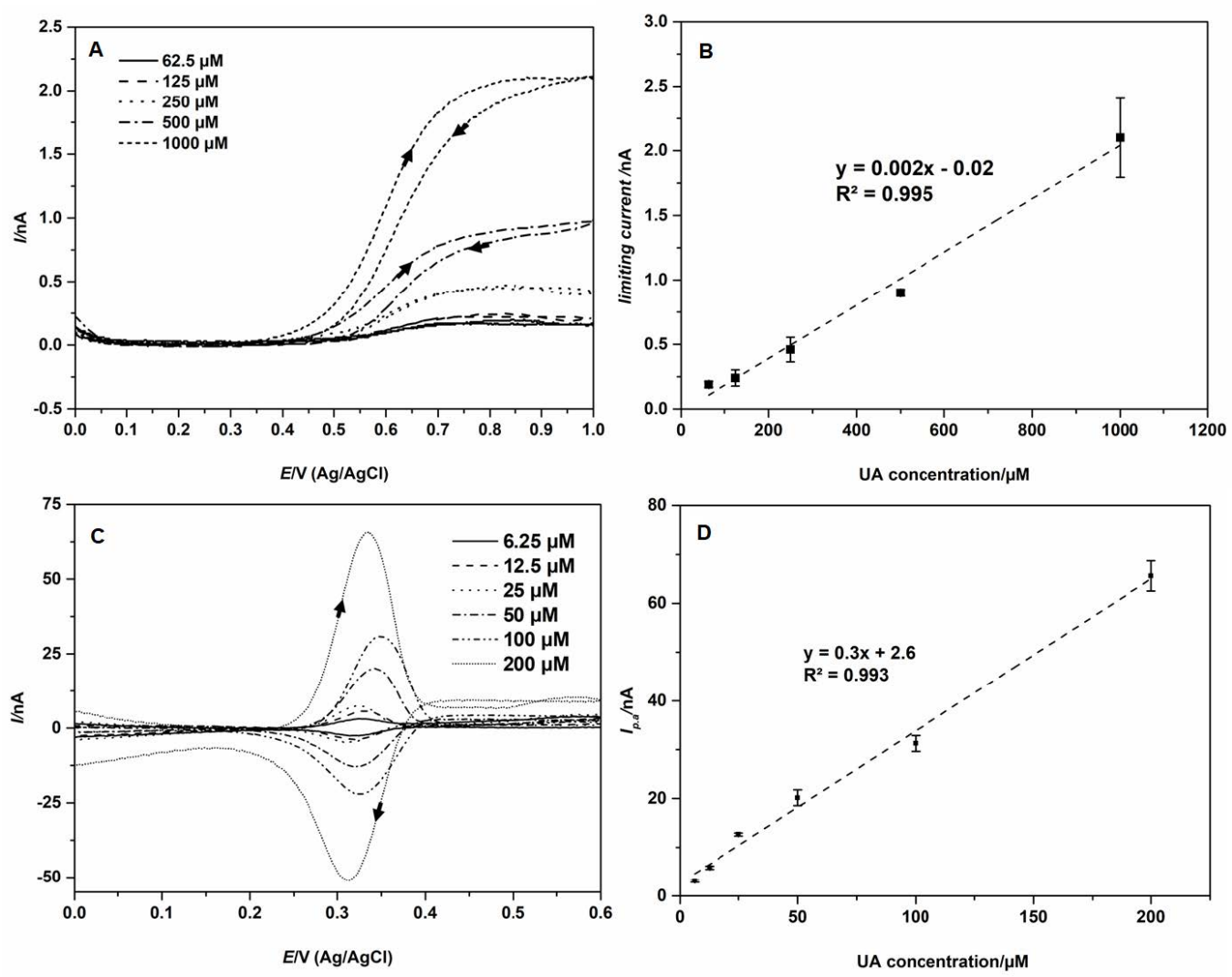
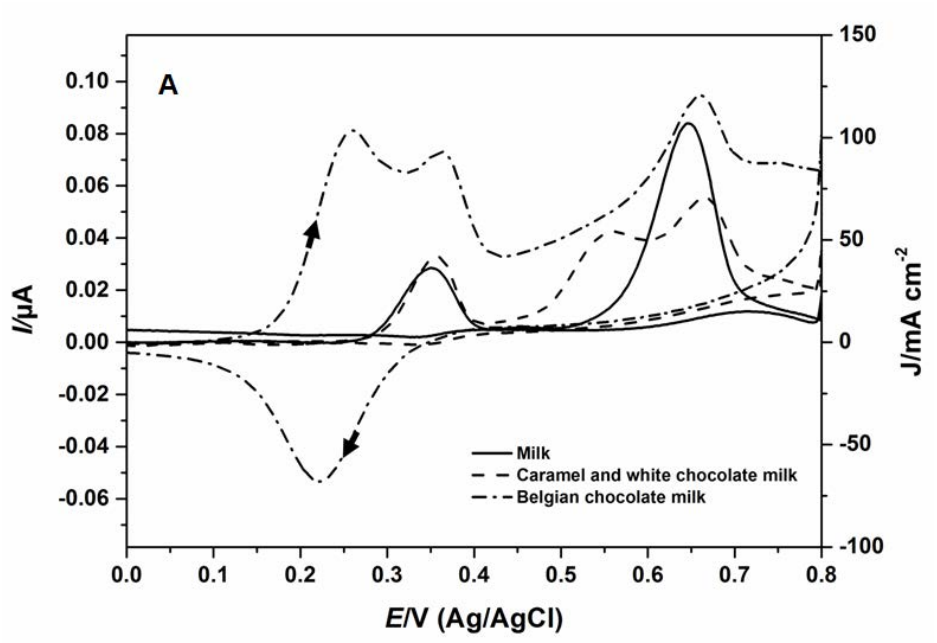


Figure 4



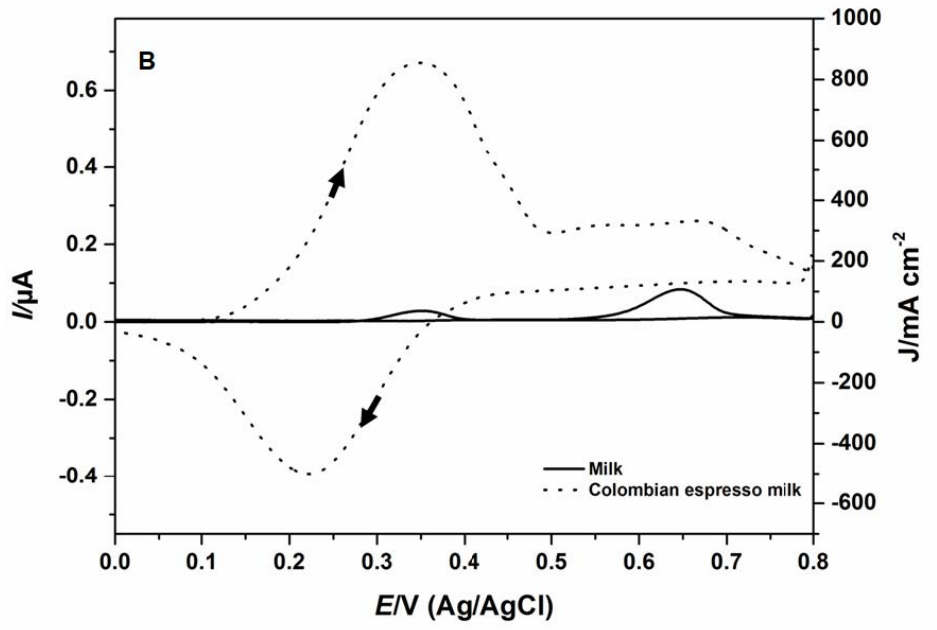


Table of Materials

Click here to access/download;Table of Materials;Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number
Acetonitrile	Baker Analyzed HPLC Ultra Gradient Solvent	75-05-8
Alumina polishing pad	BASi, USA	MF-1040
Belgian chocolate milk	Puhoi Valley dairy company, Auckland, NZ	_
Caramel/white chocolate milk	Puhoi Valley dairy company, Auckland, NZ	_
CH instrument	CH instruments, Inc. USA	_
Counter electrode	BASi, USA	MW-1032
Disodium hydrogen phosphate (Na_2HPO_4, $2H_2O$)	Scharlau Chemie SA, Barcelona, Spain	10028-24-7
DURAN bottle	University of Auckland	_
Electrochemical cell	BASi, USA	MF-1208
Electrode Polishing Alumina Suspension	BASi, USA	CF-1050
Espresso milk	Puhoi Valley dairy company, Auckland, NZ	_
3,4-Ethylenedioxythiophene (EDOT), 97%	Sigma-Aldrich	126213-50-1
FEI ESEM Quanta 200 FEG	USA	_
Gold microelectrode	BASi, USA	MF-2006
Lithium perchlorate, ACS reagent, ≥95%	Sigma-Aldrich	7791-03-9
Micropipettes	Eppendorf	_
MilliQ water	Thermo Scientific, USA	_
Propylene carbonate, Anhydrous, 99.7%	Sigma-Aldrich	108-32-7
Reference electrode	BASi, USA	MF-2052
Replacement glass polishing plate	BASi, USA	MF-2128
Sodium dihydrogen phosphate (NaH_2PO_4, $1H_2O$)	Sigma-Aldrich	10049-21-5
Sodium hydroxide pearls, AR	ECP-Analytical Reagent	1310-73-2
Sodium perchlorate, ACS reagent, ≥98%	Sigma-Aldrich	7601-89-0
Sulfuric acid (98%)	Merck	7664-93-9
Uric acid	Sigma-Aldrich	69-93-2
Whole milk	Anchor dairy company, Auckland, NZ	

Comments/Description

HPLC grade
tan/velvet color

Buy from local supermarket

Buy from local supermarket
Model CHI660E

7.5 cm long platinum wire (0.5 mm diameter) auxiliary/counter electrode, 99.95% purity

Weigh 17.8 g
The glasswares were made locally at the University of Auckland
5-15 mL volume, glass
7 mL of 0.05 µm particle size alumina polish

Buy from local supermarket
Take 10.68 µL from bottle
SEM equipped with a Schottky field emission gun (FEG) for optimal spatial resolution. The instrument can be used in high vacuum mode (HV), low-vacuum mode (LV) and the so called ESEM (Environmental SEM) mode.
Working electrode (10 µm diameter)
Make 0.1 M solution
10-100 µL and 100-1000 volumes
18.2 MΩ/cm at 25°C, Barnstead Nanopure Diamond Water Purification System
Take 20 mL from bottle
Silver/silver chloride (Ag/AgCl) electrode to be kept in 3 M sodium chloride
Glass plate as a stand to attach the polishing pad on it
Weigh 13.8 g
Make 0.1 M solution
Make 0.1 M solution
Make 0.5 M solution
Make 1 mM solution

Blue cap milk, buy from local supermarket

Electrochemical Preparation of Poly(3,4-Ethylenedioxythiophene) Layers on Gold Microelectrodes for Uric Acid Sensing Applications

3 May 2021

Dear Editor,

We would like to thank you and the reviewers for the valuable and useful comments given about our manuscript.

We have made the following corrections/modifications and additions to our manuscript. We also addressed the specific comments raised by editor and reviewers and detailed responses are listed out in the following table.

No.	Comments from the editor	Corrections Made
1	Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please use American English throughout.	All the manuscript has been checked in terms of grammar and spelling issues. American English has been used throughout the whole manuscript.
2	Please provide an email address for each author.	Email addresses of authors have been added.
3	Please ensure all abbreviations are described during the first-time use.	All abbreviations have been described in the first use.
4	Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and 1-inch margins on all side.	The manuscript format has been adjusted for paragraph indentation and line spacing. A single line space has been considered between each step, substep, and note in the protocol section. Calibri 12 points and 1 inch margin have been used in the manuscript.
5	JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of	All trademarks and registered symbols have been removed. All chemicals, materials, and commercial products have been referenced in the table of materials and reagents.

	Materials and Reagents. For example: MilliQ, Barnstead Nanopure Diamond Water Purification System, Thermo Scientific, USA, DURAN® bottle, BASi, MF-1040, BASi, MF-2128, BAS CF- 1050, CH instrument (Model CHI660E, CH instruments, Inc. USA), FEI ESEM Quanta 200 FEG, USA, etc.	
6	Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.	All the numberings have been adjusted.
7	Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."	All verbs in protocol section has been written in imperative tense.
8	Please ensure you answer the "how" question for each step, i.e., how is the step performed? This can be done by including button clicks in the software, knob turns, mechanical actions, command lines, etc.	The methods have been clarified and explained in detail.
9	How do you test the milk samples?	At section 1.6 and section 3 of the protocol, the method of testing milk samples along with the data analysis method have now been clearly explained.
10	How do you perform cyclic voltammetry?	In section 3, the CV method has been explained in detail for each sample/solutions with all different parameters that has been used.
11	Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a	The suggested issues has been amended and some notes have been omitted or transferred to the discussion section.

	particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.	
12	There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.	3 pages have been highlighted in yellow color.
13	Please revise the following lines to avoid overlap with previously published work: 208 -210, 212-213, 229-231, 264-268, 281-283, 285-289, 292-293.	The following lines have been revised and amended in the manuscript, shown using track changes: 1) Lines 208-210 (i.e. lines 371-373 in revised manuscript) 2) Lines 212-213 (i.e. lines 383-385 in revised manuscript) 3) Lines 229-231 (i.e. lines 403-405 in revised manuscript) 4) Lines 264-268 (i.e. lines 438-442 in revised manuscript) 5) Lines 281-283 (i.e. lines 456-460 in revised manuscript) 5) Lines 285-289 (i.e. lines 462-465 in revised manuscript) 6) Lines 292-293 (i.e. lines 470-471 in revised manuscript)
14	Please ensure the results are described in the context of the presented technique - you performed an experiment how did it help you to conclude what you wanted to and how is it in line with the title.	The title has been modified based on the results obtained.
15	Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”	RightsLink Printable License has been uploaded to my account as a PDF file together with the Order Certificate from John Wiley and Sons.
16	Please sort the materials table in alphabetical order.	The material table has been updated alphabetically, and more chemicals and instruments have been added to it.

NO.	Comments from the reviewer #1	Corrections Made
1	"for Sensing Applications" in the title is not justified because this work presents the preparation of the PEDOT-gold electrodes and not the applications. To be convincing, the figure 4 should present comparatively the sample analysis with the CVs using gold electrode and gold-PEDOT electrode.	Thanks for your comments. To show sensing applications of the PEDOT layer deposited on the gold microelectrode surface, uric acid was analysed both in standard solutions and in real samples such as milk and flavored milks. Figure 3 and 4 clearly shows the sensing application of the modified electrode. However, we no longer have access to the equipment and lab resources to be able compare different milk CVs on PEDOT-gold microelectrodes with bare gold microelectrodes.
2	The setup design is missing although most of the used parameters are given.	The set-up design has been explained in detail in section 3 of the protocol.
3	Please insert the characteristics of the gold microelectrode at the line 179 " 2. Pre-treatment of gold microelectrode 1. Polish the gold microelectrode" (diameter and thickness).	The thickness and diameter of the gold microelectrode have been added to section 2 of protocol, line 272.
4	At line 60 it is mentioned:"good optical transparency". What this means? We know that PEDOT is dark-blue to black.	The line 68 in revised manuscript has been amended. PEDOT has black colour, but in practice its optical absorption coefficient specially in the form of PEDOT:PSS is low in the visible spectral region at wavelengths of 400–700 nm, indicating its optical transparency in the visible part of the electromagnetic spectrum, and its functionality as a transparent electrode[*]. [*]Reference: Laskarakis, A., Karagkiozaki, V., Georgiou, D., Gravalidis, C., & Logothetidis, S. (2017). Insights on the Optical Properties of Poly(3,4-Ethylenedioxythiophene): Poly(styrenesulfonate) Formulations by Optical Metrology. *Materials (Basel, Switzerland)*, *10*(8), 959. https://doi.org/10.3390/ma10080959
5	Please also clarify: "moderate band gap, low oxidation potential … electrochemical properties and environmental stability"	Band gaps or energy gaps are regions where charges cannot exist. PEDOT has a polaronic conduction band, which decreases the band gap energy, making the polymer conductive. PEDOT is electroactive and needs lower potentials to start oxidizing. It is also stable over the time after being synthesized.
6	For clarity, in the figure 1 should be specified on every cyclic voltammogram diagram the type of electrolyte: aqueous (a-b) or organic (c-d).	The type of electrolytes have now been written on every CV in Figure 1.

NO.	Comments from the reviewer #3	Corrections Made
1	Compared with other methods, the advantages of generating PEDOT using two different electrolytes should be further elucidated. In the introduction, different methods to form PEDOT have been mentioned, like generating free radical cations to react with other free radical cations or monomers to form insoluble PEDOT chains or using an electrolyte, such as BMIMBF4. Besides, the authors said: "However, the PEDOT polymer is insoluble in most organic or aqueous solvents." or "…can be solubilized in chloroform, acetone, acetonitrile and other organic solvents, but it is still insoluble in water. And the author also pointed out some shortcomings of these methods: "The resulting electrodeposited films are influenced by different controlling factors such as electrolyte, electrode set-up, deposition time, solvent temperature etc" . However, neither the superiority of the methods proposed in this paper or whether these methods solved the problems mentioned above were not declared. It is notable that the "superiority" not only means electropolymerization of PEDOT using an organic or aqueous solution but refers to the advantages of the methods or the synthetic product, like simpler steps, rougher surfaces, and better sensitivity etc.	This section of introduction has been revised and clarified in the manuscript. The parts talking about PEDOT formation: "different methods to form PEDOT have been mentioned, like generating free radical cations to react with other free radical cations or monomers to form insoluble PEDOT chains or using an electrolyte, such as BMIMBF4" are actually explaining the mechanism of action of EDOT oxidation after potential is applied. The sentences due to solubility of PEDOT or EDOT have been revised to clarify the point better. The advantages or disadvantages of both aqueous and organic electrolytes have been explained in the results section.
2	Are the results of UA concentration that PEDOT sensor measured in real samples contented with those in standard solution In line 275, the authors said: "The anodic peak current for UA in regular milk at 0.35 V (vs. Ag/AgCl) using the	HPLC as a conventional method has also been used by our group for a real-time quantification of uric acid. Our previous published paper clearly compares the efficacy of glassy carbon sensor with an HPLC method. For example, The UA concentration in blue cap milk from Anchor company was found to be about 86 µM, while for the same milk sample this value was about 81 µM, showing a 5% average difference.

	PEDOT microelectrode is about 28.4 nA, which is equivalent to 82.7 μM using the equation of the calibration curve in Figure 3d". The author also compared these results with those measured by PEDOT-modified glassy carbon macroelectrodes, proving that the surface-modified Au microelectrodes could be used to test UA in real milk samples and show a more sensitive response than macroelectrodes. But the comparison between the two types of electrodes was insufficient to prove the value tested by the PEDOT sensor to be the real UA concentration of the milk samples. It is recommended that a commercial or commonly used method is needed to measure the concentration of UA as the ground truth.	
3	In step 3 (electropolymerization of PEDOT by cyclic voltammetry technique), the parameters of CV scan are not exactly the same in organic and aqueous solutions. However, only the parameters in organic solvents are described in the protocol.	In the revised manuscript at section 3, all the parameters for different analysis have now been included.
4	As the procedures of preparing target analyte solutions and milk samples are given, the steps of applying the PEDOT modified Au microelectrode to measure the UA concentration in standard solutions or milk samples should not be omitted.	This steps have been added in section 3 of the protocol at section 3.4.5 and 3.4.6.
5	There is a mistake in punctuation. In line 69, after the "ect.", it is no need for an additional full stop.	In lines 78, the full stop after “etc.” has been removed.
6	It is better to add the abbreviation "(LiClO4)" following the "lithium perchlorate" in line 101.	The abbreviation of lithium perchlorate has been added to the line 125 in the revised manuscript.
7	In line 121, considering that "Milli-Q" is a brand name, it is better to use	The “deionized water” has been added to line 175 in the revised manuscript.

	deionized water to replace it, like "deionized water (Milli-Q)".	
8	The expression of "PBS buffer solution" is not correct because PBS is the acronym of phosphate buffer saline. And in this paper, it is made of sodium dihydrogen phosphate and disodium hydrogen phosphate. It would be better to use "PBS" instead of "PBS buffer solution".	Since we have used phosphate buffer without saline, so for clarification of the method the “PBS buffer solution” has been replaced by “phosphate buffer solution”.
9	To identify the ingredients of milk and consider the reproducibility of the experiment, the authors need to provide the manufacturers of different milk samples.	The manufacturer name for milk samples has been added to the table of materials.
10	There is a clerical error. In line 242, fig.2c and fig.2d are described as "in the organic solution after 8 cycles", but in the figure captions, it becomes "prepared in organic solution after 4 cycles of electropolymerization".	In line 417 of the revised manuscript, the typo mistake has been corrected, with 8 cycles replaced by 4 cycles.

Following revision of the manuscript taking into account all the comments and suggestions of the reviewers, it is hoped that the quality of the manuscript has improved and can now be considered for publication.

Sincerely,

Dr. Mahsa Motshakeri, Prof. Anthony Phillips, and Prof. Paul Kilmartin,
School of Chemical Sciences,
University of Auckland

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May 13, 2021

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Lead author	Mahsa Motshakeri
Title of targeted journal	journal of visualized experiments (JOVE Method)
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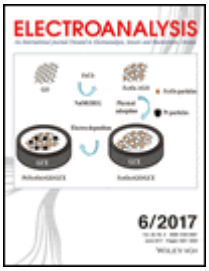

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