

# Journal of Visualized Experiments

## Manufacturing of a Nafion coated reduced graphene oxide/polyaniline chemiresistive sensor to monitor the pH in real time during microbial fermentation --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58422R2
Full Title:	Manufacturing of a Nafion coated reduced graphene oxide/polyaniline chemiresistive sensor to monitor the pH in real time during microbial fermentation
Keywords:	Reduced graphene oxide, Polyaniline, chemiresistor, potentiometric pH sensor, microsensor, Bacterial fermentation
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Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
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To **Nandita Singh**

(Senior science editor of JoVE journal)

2018-05-03

**“Polyaniline functionalized electrochemically reduced graphene oxide chemiresistive sensor to monitor the pH in real time during microbial fermentations”**

Dear Editor,

Please consider the paper entitled **“Polyaniline functionalized electrochemically reduced graphene oxide chemiresistive sensor to monitor the pH in real time during microbial fermentations”** for publication as a invited article in JoVE. In this paper we report the engineering of a solid state micro pH sensor based on polyaniline functionalized electrochemically reduced graphene oxide deposited on interdigitated gold electrode with 100  $\mu\text{m}$  interfinger spacing. We think that the manufacturing and testing of the pH electrode in a bacterial fermentation is a nice topic for a video paper. The different techniques that are used in the paper become more meaningful if you can show the reader what we have actually been doing. This work was published in “Sensors and Actuators B: chemical”. We asked the publisher of this journal for permission to reuse the graphs from that paper in this video paper as well. If the publisher does not agree we will make some new graphs from different experiments. Of course, the data will lead to the same conclusions.

Thank you for considering the paper for publication in JoVE.

Sincerely,  
Prof. Dr. Gert Jan William Euverink

**TITLE:**

**Manufacturing of a Nafion-coated, Reduced Graphene Oxide/Polyaniline Chemiresistive Sensor to Monitor pH in Real-time During Microbial Fermentation**

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**KEYWORDS:**

Reduced graphene oxide, polyaniline, chemiresistor, potentiometric pH sensor, microsensor, bacterial fermentation

**SUMMARY:**

Here, we report the protocol for the fabrication of a Nafion-coated, polyaniline-functionalized, electrochemically reduced graphene oxide chemiresistive micro pH sensor. This chemiresistor-based, solid-state micro pH sensor can detect pH changes in real-time during a *Lactococcus lactis* fermentation process.

**ABSTRACT:**

Here, we report the engineering of a solid-state micro pH sensor based on polyaniline-functionalized, electrochemically reduced graphene oxide (ERGO-PA). Electrochemically reduced graphene oxide acts as the conducting layer and polyaniline acts as a pH-sensitive layer. The pH-dependent conductivity of polyaniline occurs by doping of holes during protonation and by the dedoping of holes during deprotonation. We found that an ERGO-PA solid-state electrode was not functional as such in fermentation processes. The electrochemically active species that the bacteria produce during the fermentation process interfere with the electrode response. We successfully applied Nafion as a proton-conducting layer over ERGO-PA. The Nafion-coated electrodes (ERGO-PA-NA) show a good sensitivity of 1.71  $\Omega$ /pH (pH 4 - 9) for chemiresistive sensor measurements. We tested the ERGO-PA-NA electrode in real-time in the fermentation of *Lactococcus lactis*. During the growth of *L. lactis*, the pH of the medium changed from pH 7.2 to pH 4.8 and the resistance of the ERGO-PA-NA solid-state electrode changed from 294.5  $\Omega$  to 288.6  $\Omega$  (5.9  $\Omega$  per 2.4 pH unit). The pH response of the ERGO-PA-NA electrode compared with the response of a conventional glass-based pH electrode shows that reference-less solid-state microsensor arrays operate successfully in a microbiological fermentation.

**INTRODUCTION:**

pH plays a vital role in many chemical and biological processes. Even small changes in the pH value alter the process and adversely affect the outcome of the process. Hence, it is necessary to monitor and control the pH value during every stage of experiments. The glass-based pH electrode has been successfully used to monitor pH in many chemical and biological processes, although the use of a glass electrode poses several limitations to measuring pH. The glass-based pH electrode is relatively large, fragile, and small leakages of the electrolyte into the sample are possible. Furthermore, the electrode and electronics are relatively expensive for applications in 96-well screening fermentation systems. Moreover, the electrochemical sensors are invasive and consume the sample. Hence, it is more advantageous to use non-invasive, reference-less sensors.

Nowadays, miniaturized reaction systems are favored in many chemical engineering and biotechnology applications as these microsystems provide enhanced process control, along with many other advantages over their macro system analogs. To monitor and control the parameters in a miniaturized system is a challenging task as the sizes of the sensor to measure, for instance, pH and O<sub>2</sub>, need to be minimized as well. The successful production of microreactors for biological systems require different kinds of analytical tools for process monitoring. Hence, the development of smart microsensors plays a significant role in carrying out biological processes in microreactors.

Recently, there have been several attempts to develop smart pH sensors using chemiresistive sensing materials like carbon nanotubes and conducting polymers<sup>1</sup>. These chemiresistive sensors require no reference electrode and are easy to integrate with electronic circuits. Successful chemiresistive sensors make it possible to produce smart sensors that are cost-effective and easy to manufacture, require a small volume for testing, and are non-invasive.

Here, we report a method to develop an electrode with polyaniline-functionalized, electrochemically reduced graphene oxide. The chemiresistive electrode operates as a pH sensor during an *L. lactis* fermentation. *L. lactis* is a lactic-acid-producing bacterium used in food fermentation and food preservative processes. During fermentation, the production of lactic acid lowers the pH, and the bacterium stops growing at a low pH<sup>2-4</sup>.

A fermentation medium is a complex chemical environment that contains peptides, salts, and redox molecules which tend to interfere with the sensor surface<sup>5-9</sup>. This study shows that a pH sensor based on chemiresistive material with a proper surface protection layer could be used to measure pH in this kind of complex fermentation media. In this study, we successfully use Nafion as the protection layer for polyaniline-coated, electrochemically reduced graphene oxide to measure the pH in real-time during an *L. lactis* fermentation.

## PROTOCOL:

### 1. Preparation of Graphite Oxide

Note: Graphite oxide (GO) is prepared according to Hummers' method<sup>10,11</sup>.

1.1. Add 3 g of graphite into 69 mL of concentrated H<sub>2</sub>SO<sub>4</sub> and stir the solution until the graphite has completely dispersed. Add 1.5 g of sodium nitrite and leave it for 1 h while stirring. Then, place the container in an ice bath.

1.2. Add 9 g of potassium permanganate into the dispersion and remove the container from the ice bath. Allow the solution to warm up to room temperature.

1.3. First, add 138 mL of distilled water dropwise. Then, continue to add 420 mL of distilled water. Maintain the temperature at 90 °C for 15 min using a hotplate. Add 7.5 mL of 30% hydrogen peroxide to the dispersion.

1.4. Collect the product by centrifugation at 10,000 x g for 20 min and discard the supernatant solution. Wash the pellet 4x with warm double-distilled water and 2x with a 10% HCl (v/v) solution. Finally, wash it 2x with ethanol and dry it at 50 °C in the oven.

## **2. GO-deposited Electrode Preparation**

2.1. Disperse 10 mg of GO in 10 mL of water and then sonicate it in an ultrasonic bath for 6 h.

2.2. Remove the unexfoliated GO flakes by centrifugation for 30 min at 2,700 x g. Discard the solid particles after centrifugation and use the supernatant for further experiments.

Note: We used this exfoliated GO flakes dispersion as the stock solution.

2.3. Dilute the GO stock solution two-fold. Always prepare a fresh GO working solution from the stock solution.

2.4. Add 2 µL of the GO working solution on top of an exposed interdigitated gold electrode (**Figures 1A and 2**). After drop casting, dry the electrode at room temperature for 12 h. This is the GO-deposited electrode.

## **3. Reduction of GO to Electrochemically Reduced Graphene Oxide**

3.1. Insert the electrode in the polydimethylsiloxane (PDMS) electrode holder (bottom piece). Place the other part of the electrode holder, which serves as a solution reservoir, on top of the electrode as shown as in **Figures 1A - 1C**. Assemble the holders by clipping the two parts together using two paper clips. Make sure that the PDMS holder does not cover the GO-deposited electrode part.

3.2. Pipet 300 µL of 0.2 M phosphate buffer (pH 7) in the reservoir. Then, place the reference and the counter electrode in the solution in such a way that the electrodes are placed close to the surface of the GO film, as shown in **Figure 1C**. This set-up serves as an electrochemical cell to perform electrochemical reduction of GO and for polyaniline deposition.

3.3. Connect the electrodes with the potentiostat connected to a computer for data acquisition. Use cyclic voltammetry for the electrochemical reduction: select 0 to -1.2 V as a potential range and 50 mV/s as the scan rate. Cycle the voltage over the electrode between 0 to -1.2 V 10x (**Figure 3**).

3.4. After the experiment, remove the electrode from the holder and repeatedly wash it with double-distilled water. Then, dry the electrode in an oven at 101 °C for 12 h.

3.5. When the electrode is dry, remove the electrode from the oven and allow it to cool down to room temperature. Then, measure the conductivity of the electrode with a multimeter. The electrode is now referred to as an electrochemically reduced graphene oxide (ERGO) electrode.

#### **4. Polyaniline Functionalization of the ERGO Electrode**

4.1. Prepare 10 mM aniline monomer for the polyaniline functionalization. Dissolve 5 µL of 10 mM aniline in 5 mL of 1 M H<sub>2</sub>SO<sub>4</sub>.

4.2. For the polyaniline functionalization, add 300 µL of aniline monomer to the solution reservoir. Place the ERGO-deposited electrode into the electrode holder as described in the procedure for GO reduction.

4.3. Use cyclic voltammetry for the electropolymerization of aniline to functionalize ERGO into ERGA-polyaniline (ERGO-PA): select 0 to 0.9 V as a potential range and 50 mV/s as the scan rate. Cycle the voltage over the electrode between 0 to 0.9 V for 50x (**Figure 4**).

4.4. After the polyaniline deposition, remove the electrode and repeatedly wash it with double-distilled water. Then, dry the electrode at 80 °C in the oven for 12 h.

4.5. Remove the electrode from the oven and allow it to cool down to room temperature before measuring the conductivity of the electrode with a multimeter.

4.6. Prepare a pH 5 buffer solution by adding 0.2 M NaOH to the Britton-Robinson buffer solution until pH 5 (see step 5.1). Keep the electrode in the buffer at pH 5 for 24 h.

4.6.1. To prepare a Britton-Robinson universal buffer solution, mix 0.04 mol of phosphoric acid, 0.04 mol of acetic acid, and 0.04 mol of boric acid in 0.8 L of ultrapure water. Add 0.2 M sodium hydroxide dropwise to the buffer solution until the desired pH is reached<sup>4</sup>. Add ultrapure water until the final volume is 1 L.

#### **5. ERGO-PA Electrode Testing at Different pH (Pre-calibration Before Nafion Coating)**

5.1. After conditioning the electrode in a pH 5 buffer solution, measure the resistance of the electrode in solutions of a different pH (from pH 4 to pH 9; see **Figure 5**).

5.1.1. For this measurement, dip the electrode directly into the buffer solution and connect the other part of the electrode to the computer-controlled potentiostat for data acquisition. Change the pH by titrating with 0.2 M NaOH.

5.1.2. Choose chronopotentiometry or amperometry i-t curve from the list of techniques and apply a 100 mV potential difference to the electrode.

Note: The potentiostat measures the current against time. The software controlling the potentiostat provides a graphical representation of the current against time.

5.1.3. Use Ohm's law (resistance equals voltage divided by current) to calculate the resistance value from the measured current and applied voltage.

5.2. After the measurements, dry the electrode at room temperature for 12 h.

## 6. Preparation of the Nafion-coated ERGO-PA Electrode

6.1. Add 5  $\mu$ L of 5 wt% Nafion on top of the ERGO-PA electrode and dry the electrode at room temperature for 12 h.

6.2. After the Nafion coating, keep the electrode in the buffer solution at pH 5 for 24 h before pH measurements.

6.3. After conditioning in pH 5, remove the Nafion-coated ERGO-PA electrode (ERGO-PA-NA) and measure the resistance of the electrode from pH 4 to pH 9 as mentioned in section 5.1 (Figure 6).

## 7. Preparation of *L. lactis* Culture Medium

7.1. Add 9.3 g of M17 powder into 250 mL of ultrapure water. Slowly agitate the solution until the powder dissolves completely. Autoclave the solution at 121 °C for 15 min.

7.2. Take a 250-mL sterilized flask with a magnetic stirrer bar and add 50 mL of the sterilized M17 medium to the flask. Then, add 8 mL of autoclaved 1 M glucose solution. Inoculate the solution with 10  $\mu$ L of an *L. lactis* culture, previously grown in the same culture medium.

Note: The bacterial strain was obtained from Jan Kok, Molecular Genetics, University of Groningen.

7.3. Place the flask with the inoculated culture medium for 18 h on a magnetic stirrer plate in an incubation oven at 30 °C while stirring and monitor the pH.

## 8. Testing of the ERGO-PA-NA pH Response in an *L. lactis* Fermentation Experiment

8.1. Place the ERGO-PA-NA electrode into the *L. lactis* culture and close it with a cotton plug. Then, place the set-up into the thermostat at 30 °C to grow *L. lactis*.

8.2. Apply 100 mV to the electrode and measure the current against time.

8.3. Take 0.5-mL samples at different time points (see, for instance, **Figure 7**) to measure off-line the optical density at 600 nm and the pH with a conventional glass electrode. Continue the measurements until the optical density of the culture becomes constant, indicating that the bacteria are not growing anymore.

#### REPRESENTATIVE RESULTS:

The appearance of a strong reduction peak around -1.0 V (**Figure 3**) illustrated the reduction of GO to ERGO<sup>12-14,22</sup>. The intensity of the peak depends on the number of GO layers on the electrode. A thick black film completely covered the gold wires on the electrode. At that point, the two insulated gold electrodes were conductive because the GO connected the two gold electrode wires. Electropolymerization of aniline deposited a green film on the ERGO<sup>15-22</sup>. This green color is an indication of the formation of a conductive polyaniline layer on the ERGO. The conductivity of the ERGO electrode (resistance decrease) increased after the polyaniline functionalization.

When we put the ERGO-PA electrode in a solution with a pH between 4 and 9, the current value increased (**Figure 5**) due to the doping and dedoping of holes during the protonation/deprotonation process in ERGO-PA (**Figure 2**)<sup>22</sup>. The desired pH value for the measurement of the current value of the ERGO-PA electrode was obtained by titrating the Britton-Robinson buffer solution with 0.2 M NaOH. Hence, for every addition of 0.2 M NaOH, the current value of the electrode increased (**Figures 5 and 6**). The response of the electrode was immediately stable when the addition of 0.2 M NaOH stopped at a particular pH.

A thin film of proton-conductive Nafion formed after the solvent evaporated at room temperature. The conductivity of the electrode was not affected much, but a few ohms of difference in the resistance value occurred and changed the base current value of the ERGO-PA electrode. Similar to the ERGO-PA electrode, the resistance of the ERGO-PA-NA electrode changed when the pH of the buffer solution changed from 4 to 9, as shown in **Figure 6**<sup>18</sup>.

After placing the ERGO-PA-NA electrode inside the *L. lactis* culture, the current initially decreased and then took some time to reach a stable value. Once the growth of *L. lactis* started, the current of the ERGO-PA-NA decreased gradually. The decrease in current accelerated during the exponential growth-phase of *L. lactis* and reached a stable value at the end of the growth (**Figure 7**)<sup>18</sup>. The final value of the current (or resistance) is comparable to the current value of the ERGO-PA-NA electrode tested in buffer solution (pH 4 - 7), as shown in the inset of **Figure 7**.

#### FIGURE LEGENDS:



**Figure 1: Images of the bottom (left) and the top (right) part of the PDMS electrode holder. (A)** The assembled cell with (B) reference and (C) counter electrode. (D) The interdigitated gold electrode with the scale bar in centimeters.

**Figure 2: Schematic of ERGO-PA-deposited interdigitated gold electrode with a graphical representation of ERGO and PA formation.** The image also shows hole doping on ERGO-PA during protonation.

**Figure 3: Cyclic voltammetry of GO reduction with different GO concentrations at a scan rate of 50 mV/s.**

**Figure 4: Cyclic voltammetry of polyaniline deposition at a scan rate of 50 mV/s.** The first 10 scans from a total of 50 are shown. The vertical arrow marks the trend of the current increase during the scans, and the horizontal arrows mark the direction of the voltage scan.

**Figure 5: Resistance value of the ERGO-PA electrode against pH 4 to 9.**

**Figure 6: Resistance value of the ERGO-PA-NA electrode against pH 4 to 9.**

**Figure 7: Real-time continuous pH change of ERGO-PA-NA during *L. lactis* fermentation.** The inset shows the expected resistance value of ERGO-PA-NA for pH 4 - 7 measured in Britton-Robinson buffer solution.

## DISCUSSION:

It is essential that the GO layers completely cover the gold electrode wires after the deposition of GO. If the gold electrodes are not covered with GO, polyaniline will not only deposit on ERGO but also on the visible gold electrode wires directly. Deposition of polyaniline on the gold electrode wires may have implications on the performance of the electrode. After the reduction of GO to ERGO, the electrode is dried at 100 °C to strengthen the bonding between the ERGO layer and the gold electrode wires. The resistance of each electrode varies based on the number of GO layers that are deposited on the gold electrodes. Therefore, it is important to have the same concentration of GO for each electrode, and it is difficult to manufacture the electrode with a resistance in a predetermined specified range that is compatible with the measuring circuit. This limits the easy mass production of the electrodes.

The preparation of reduced graphene oxide/polyaniline by an electrochemical method has some advantages over other reported preparation methods. The electrochemical method presented here does not require strong reducing and oxidizing agents (*e.g.*, hydrazine and ammonium persulfate)<sup>23,26</sup>. In addition, the material is directly deposited on the electrode and no further processing is required, making the fabrication process faster and easier. As GO is electrochemically reduced *in situ*, a good connection between the gold and the graphene is achieved, making the pH electrode more robust.

Equilibrating the ERGO-PA electrode in a buffer with a pH between 3 and 9 before applying the Nafion improved the sensitivity of the electrode (data not shown). Omitting this step requires a soaking of the ERGO-PA-NA electrode in a buffer pH 5 for more than 24 h before use.

Furthermore, the ERGO-PA electrode must be dry before applying Nafion. A wet ERGO-PA electrode resulted in an aqueous layer between the ERGO-PA and Nafion and increased the response time of the pH sensor. The resistance or measured current of ERGO-PA-NA in solutions with a different pH varied between electrodes. This variation in resistance or current for each electrode is, most likely, caused by the difference in the number of GO layers deposited on the gold electrode wires. Just like with other pH electrodes, proper calibration of the ERGO-PA-NA electrode is necessary to obtain reliable pH values.

After placing the electrode inside the *L. lactis* culture, an initial stabilization time is necessary to obtain a constant current. In the *L. lactis* fermentation, the initial pH is 7.2. During the growth of *L. lactis*, glucose is converted into biomass and into lactic acid that acidifies the fermentation liquid. The growth stops when the pH of the fermentation medium becomes too low to support proper growth or when there is no glucose left. The current (or resistance) value of ERGO-PA-NA before and after growth are equal to the current (or resistance) value of ERGO-PA-NA previously calibrated in different buffer solutions. The initial pH and end pH of the *L. lactis* fermentation medium was confirmed using a conventional glass pH electrode.

The pH sensor can be easily manufactured in-house using cheap chemicals. The low manufacturing costs allow researchers to use this electrode in applications where a large number of pH electrodes are necessary (e.g., in a bacterial fermentation screening platform). Another application of the pH electrode is envisioned in situations where the diffusion of KCl from a conventional glass pH electrode into the measuring solution is not wanted. The pH electrode of this protocol has no internal liquids that can diffuse into the sample.

Compatibility of the chemiresistive sensor with currently available wireless electronic circuits<sup>1,27</sup> makes it possible to easily develop applications using wireless pH sensors.

#### **ACKNOWLEDGMENTS:**

The authors acknowledge the University of Groningen for financial support.

#### **DISCLOSURES:**

The authors have nothing to disclose.

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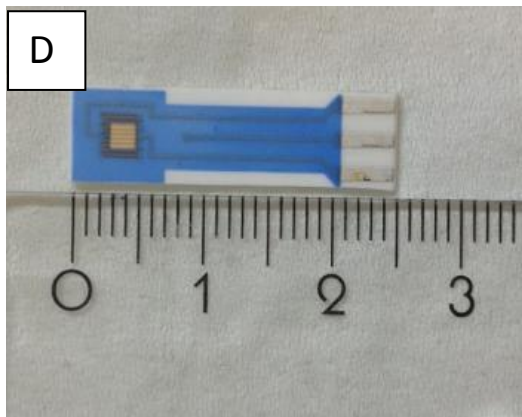
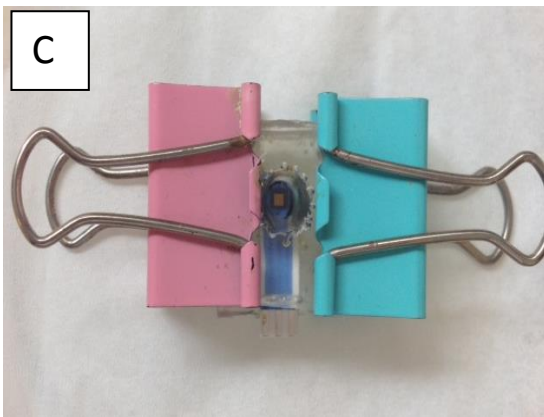
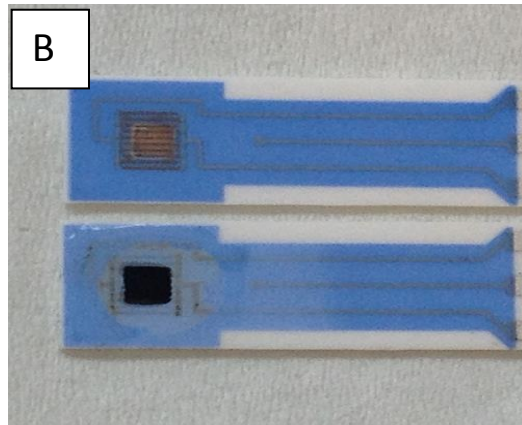
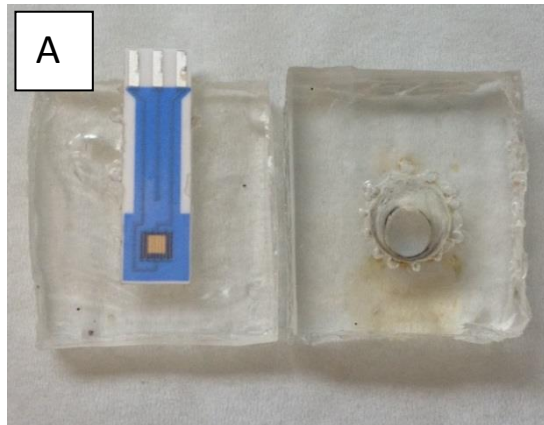


Figure 2

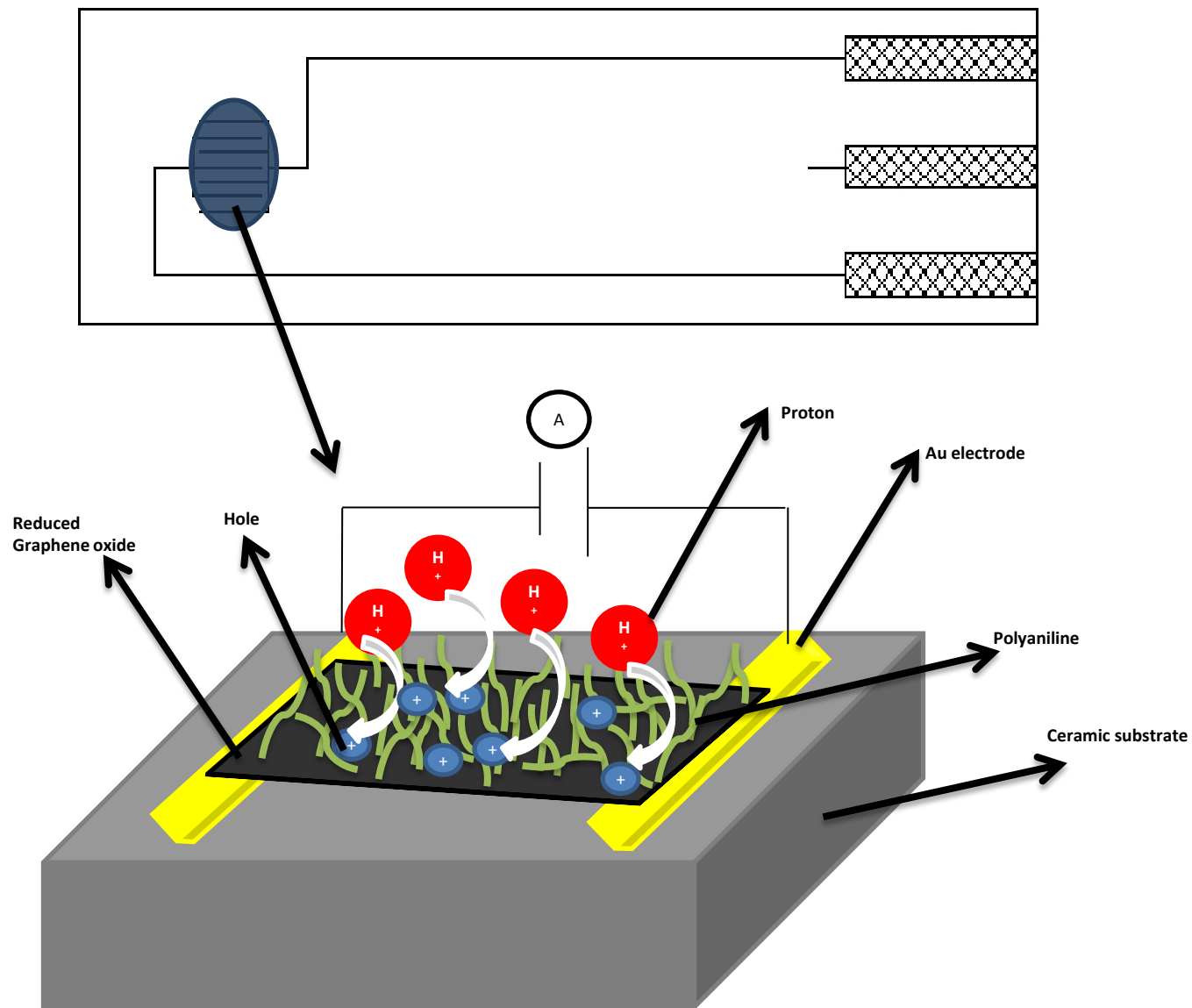


Figure 3

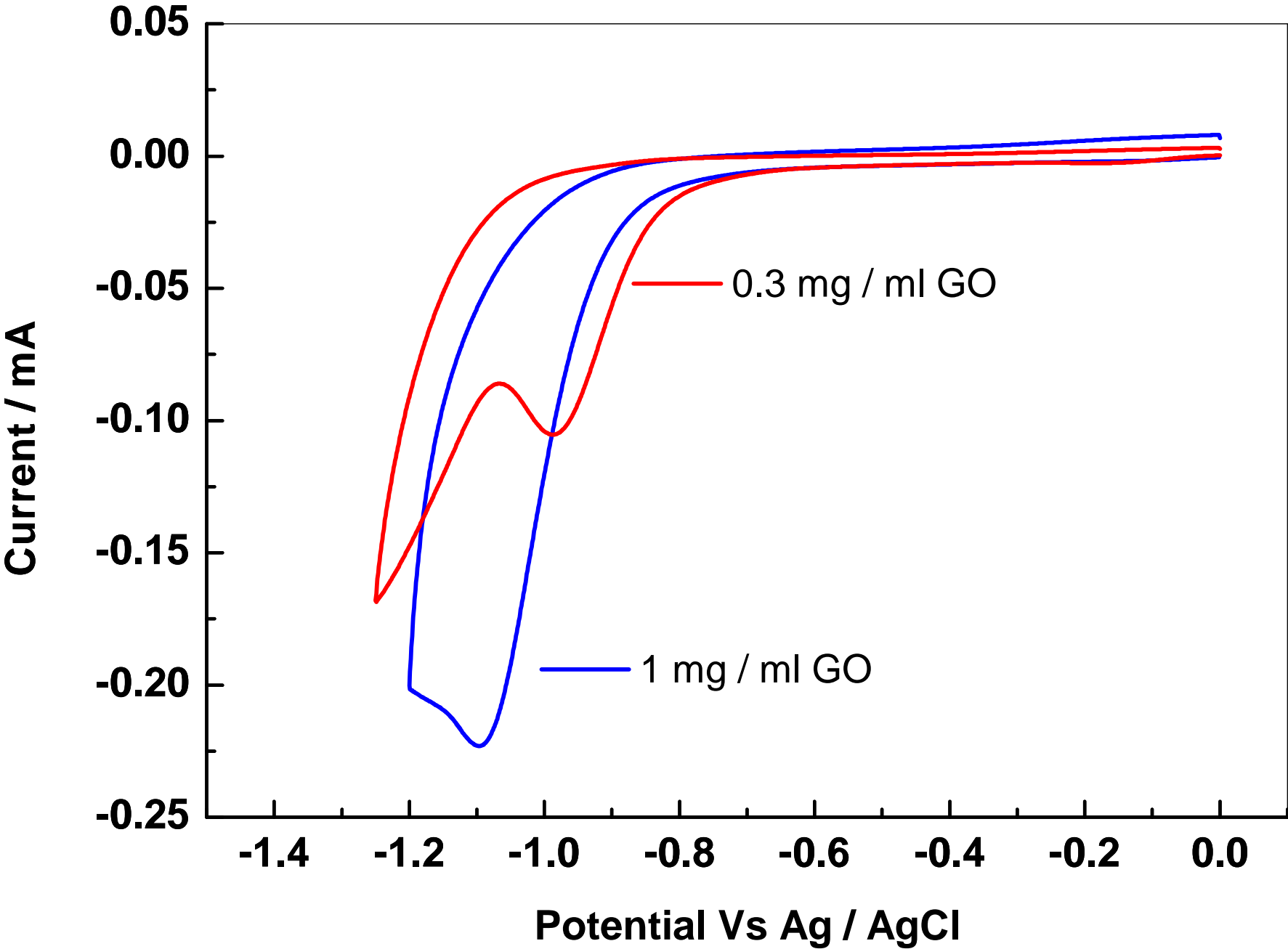


Figure 4

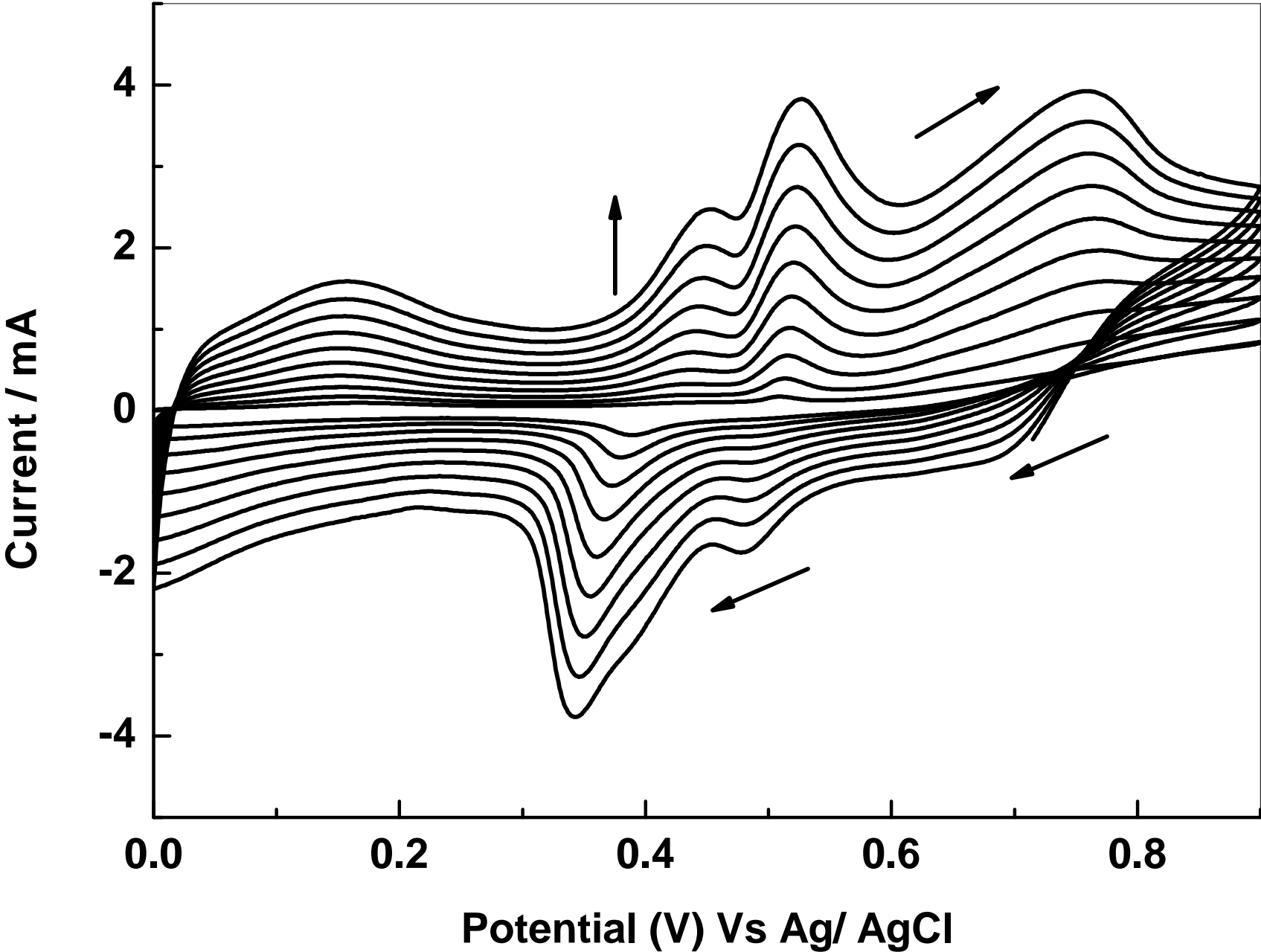




Figure 5

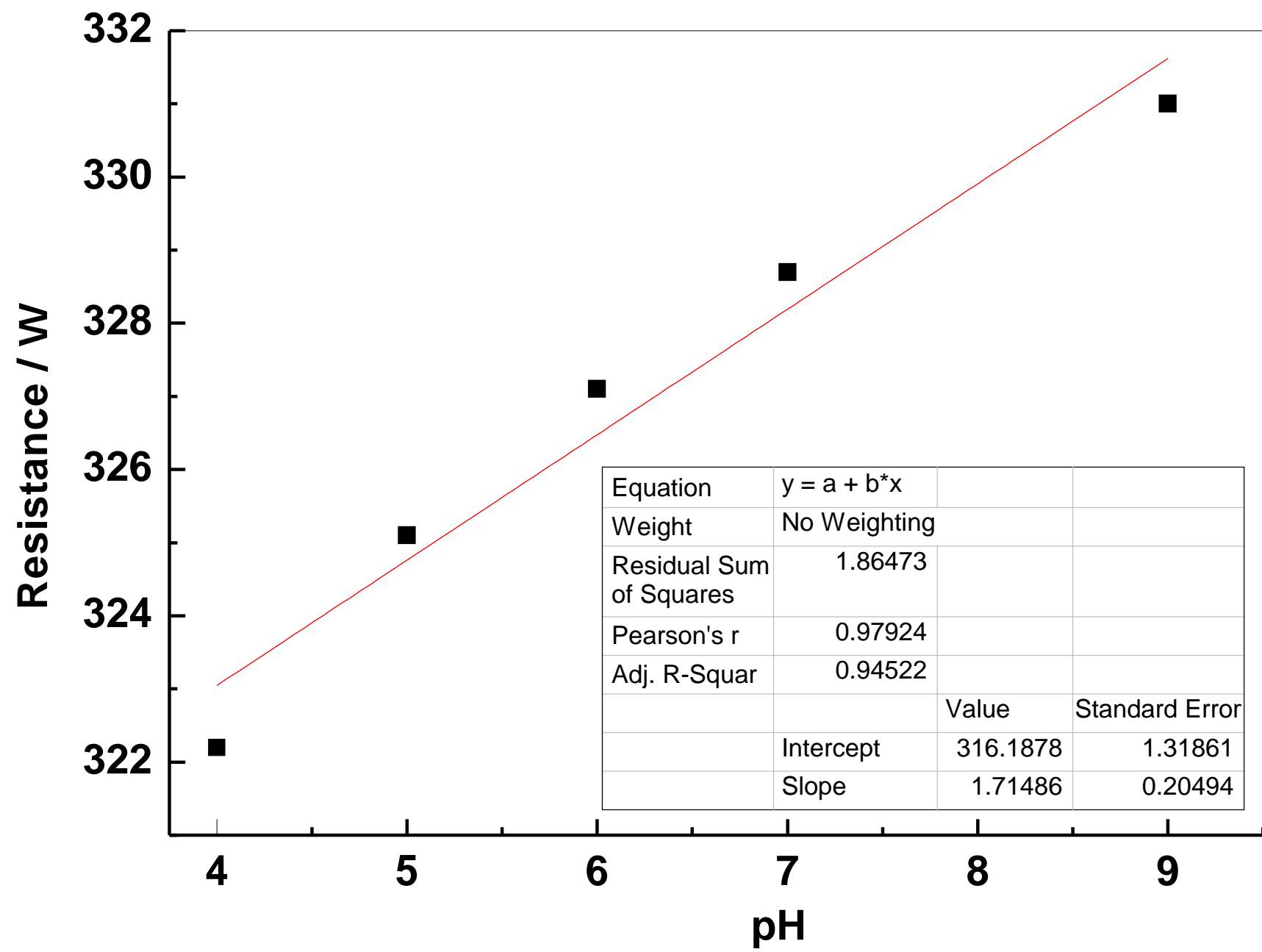


Figure 6

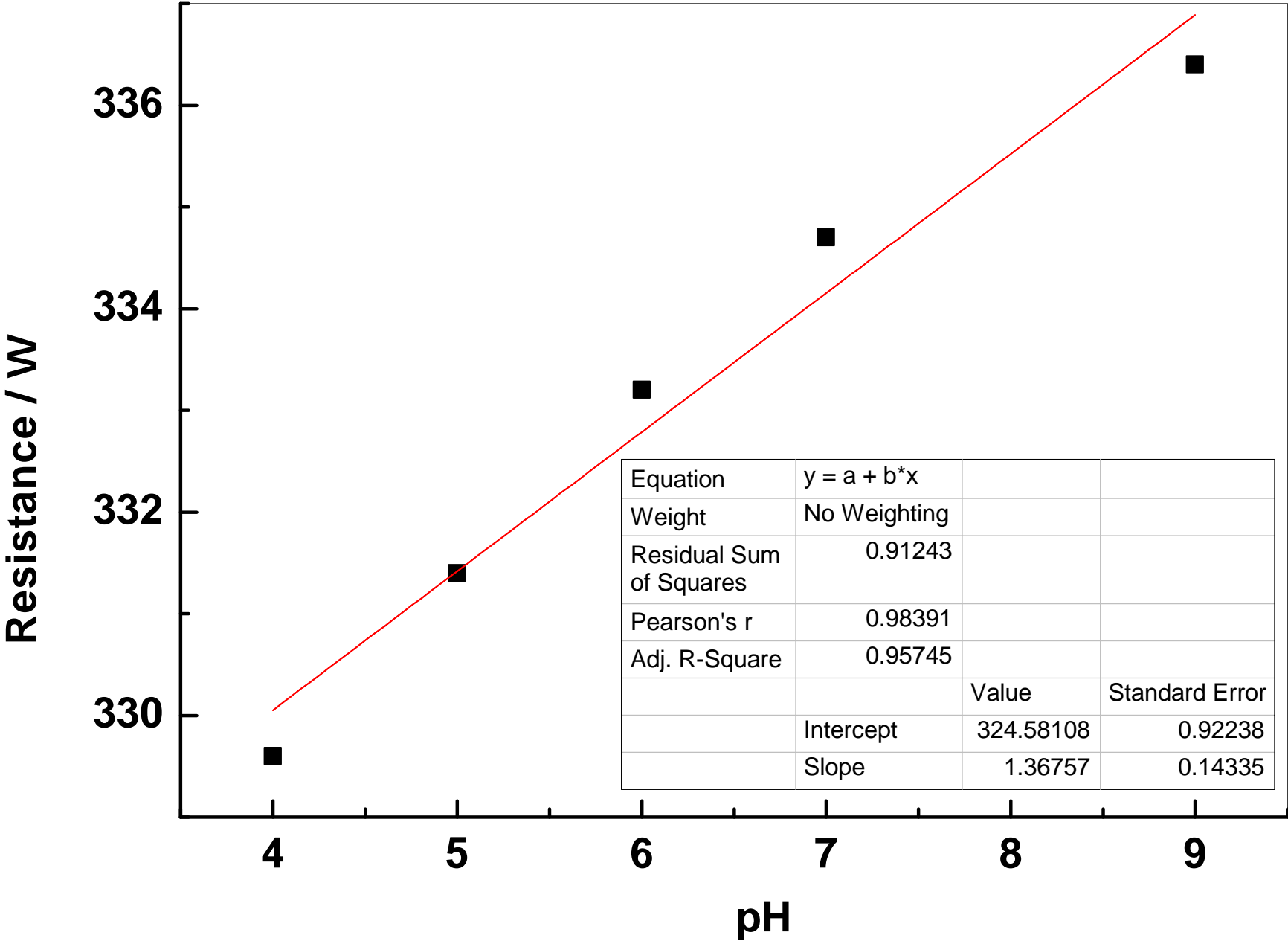
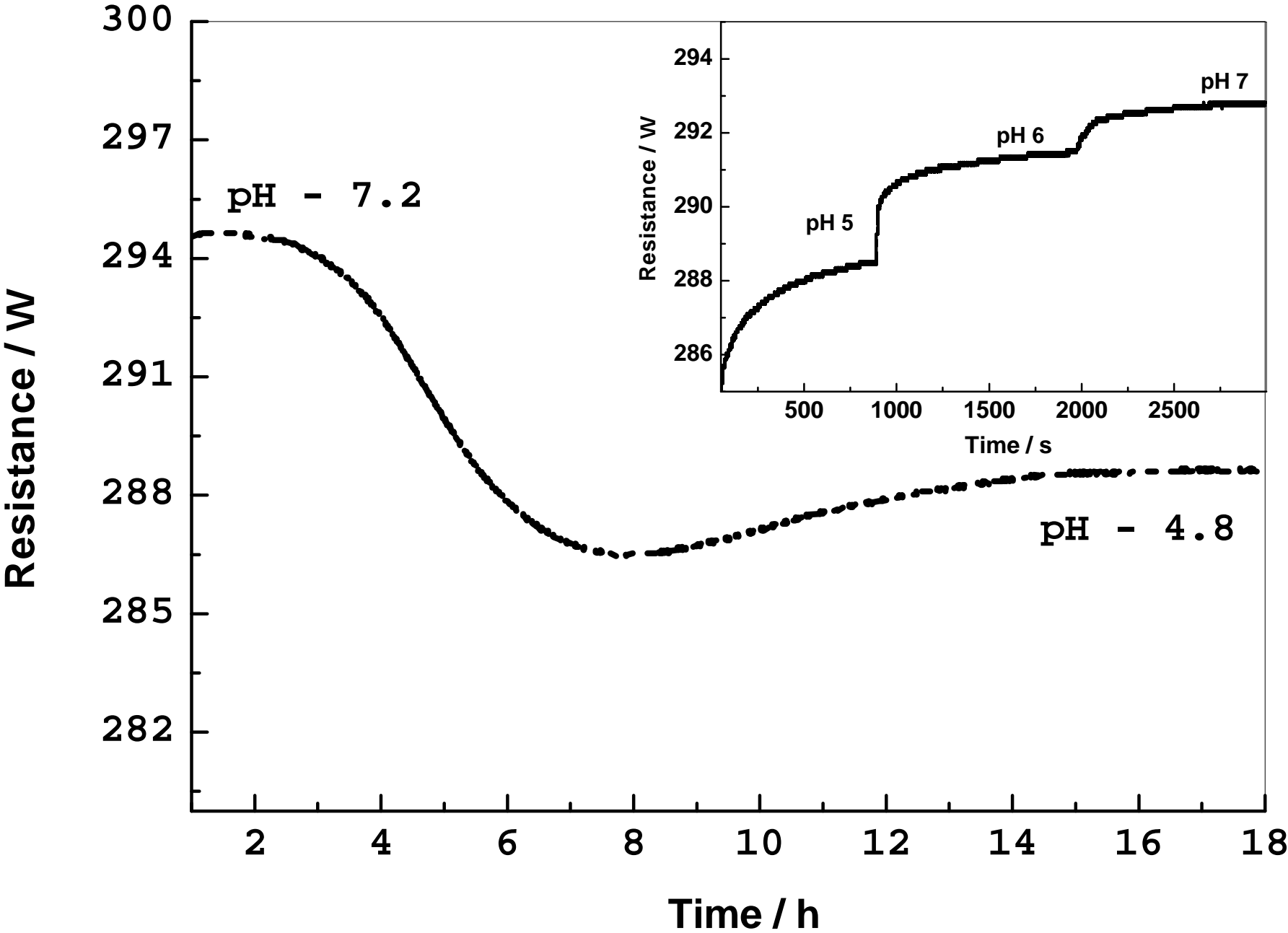


Figure 7



Name of Reagent/ Equipment	Company
Graphite flakes	Sigma Aldrich
Sulfuric acid (H <sub>2</sub> SO <sub>4</sub> )	Merck
Sodium nitrite (NaNO <sub>2</sub> )	Sigma Aldrich
Potassium permanganate (KMnO <sub>4</sub> )	Sigma Aldrich
30 % H <sub>2</sub> O <sub>2</sub>	Sigma Aldrich
HCL	Merck
Aniline	Sigma Aldrich
5wt % Nafion	Sigma Aldrich
M17 powder	BD Difco
Phosphoric acid (H <sub>3</sub> PO <sub>4</sub> )	Sigma Aldrich
Boric acid (HBO <sub>3</sub> )	Merck
Acetic acid	Merck
Sodium Hydroxide	Sigma Aldrich
Potassium dihydrogen phosphate	Sigma Aldrich
Dipostassium hydrogen phosphate	Sigma Aldrich
Au Interdigitated electrodes	BVT technology - CC1 W1
Potentiostat	CH Instruments Inc (CH-600, CH-700)



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Author(s):

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1. Please renumber references in order that they appear in the manuscript (e.g., currently reference 21 is cited before 11). Also, please don't include brackets around citations.

We have renumbered the references and excluded brackets from all the citations and used superscript formatting.

2. 3.3/6.1: Please explain a bit more about how cyclic voltammetry and chronopotentiometry will be done-e.g., are you using a computer controller?

Cyclic voltammetry and chronopotentiometry / Amperometry i-t curve measurement done by potentiostat connected with computer for data acquisition. We explained this in the manuscript.

3. 8.2: Please include a reference for *L. Lactis* preculture.

The bacterium *Lactococcus lactis* was obtained from colleagues within our university. Prof dr Jan Kok, Molecular Genetics, University of Groningen. We added this reference in the manuscript

4. 9.3: This is unclear-is the hourly sample for OD600 and the half-hourly one for pH measurement?

We rephrased 9.3 in such a way that it became less confusing.

5. Figure 1: Is the scale cm/mm?

The scale is in "CM". The scale "CM" is mentioned in the figure caption.

6. Figure 3: '0.3', not '0,3'.

The inside number from the Figure 3 is corrected as 0.3.

7. Figure 4: What is the trend with increasing scan number? Which scans are shown in this?

In figure 4 the first 10 scans are shown. The trend is indicated with an arrow.

8. Figure 5/6: Please include equations of the fits along with R-squared (or other measurement of goodness-of-fit).

We included fitting details in Figure 5 and Figure 6.

9. Figure 7: The inset is blurry (even in the original image file). Also, how exactly was the inset generated, and is the x-axis really hours? Please use 'h' if so.

We exported all figures in a PDF vector format. Thank you for the correction. The unit of the x-axis is in seconds.

.



10. Discussion: Please include more on limitations of the procedure, the significance with respect to other methods (with citations), and future applications here.

We included advantages and limitations of our electrode preparation over the reported methods in the discussion. We also added future applications of our reported pH electrode.