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

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Highlights :

- Reduced graphene oxide is prepared on interdigitated gold electrode, with 100 μm interfinger spacing, by electrochemical reduction of graphene oxide
- Reduced graphene oxide is electrochemically functionalized by polyaniline and used as referenceless chemiresistive pH sensor
- Nafion is used as protective layer for pH measurement in complex bacterial fermentation froth
- Continuous real time pH measurement in *Lactococcus Lactis* fermentation is successfully performed.

Abstract:

Here we report potentiometric and chemiresistive pH sensing performance of polyaniline functionalized Electrochemically reduced graphene oxide (ERGO-PA) to monitor pH change in *Lactococcus lactis* fermentation continuously in real time. We found that polyaniline functionalized graphene oxide as such, cannot be used in a fermentation process to monitor pH. A lot of interference was observed from electrochemically active redox species that are produced during the bacterial growth. We have successfully employed a simple Nafion

coating on top of the ERGO-PA to avoid interferences. The Nafion coated electrodes show a good sensitivity of 55 mV / pH (pH 2 - 9) and 1.71 Ω / pH (pH 4-9) for potentiometric and chemiresistive sensor measurements, respectively. Real time pH measurements of Nafion coated ERGO-PA in a *L. lactis* fermentation show that it can be used for continuous pH measurements in complex biological growth media. Effectiveness of ERGO-PA-NA solid state chemiresistive pH sensor in complex fermentation process shows that reference less solid state micro sensors can be fabricated and could be used in e.g. multi micro bioreactor screening platforms.

Keywords : Reduced graphene oxide, Polyaniline, chemiresistor, potentiometric pH sensor, microsensor, Bacterial fermentation

1. Introduction:

Nowadays, multiple micro bioreactors are attractive to use as a screening platform to study and optimize fermentation processes because of its advantage to be able to have a large number of reactors available at an affordable prize. Therefore, more fermentation conditions can be screened in comparison with traditional larger sized fermentation vessels. However, small reactors require small sensors and a large number of reactors require cheap sensors. Temperature and pH are, among others, important parameters to measure and control in a fermentation. Simple, small and cheap temperature sensors are widely available but small and cheap pH sensors are not commercially available. Therefore, pH measurements in small micro bioreactors during fermentation processes is cumbersome because both glass and ISFET based pH sensors require reference electrode which are invasive and causes possible intrusion of potassium chloride impurities which may have a big impact in the small fermentation volumes. Recently, there has been an attempt to develop referenceless chemiresistive pH electrode using CNT/conducting polymer composites [1]. The potentiometric and

conductometric pH response of CNT/conducting polymer in clear buffer solution was reported. But, studying this kind of chemiresistive materials in a complex fermentation medium poses challenges as the microorganisms in the fermentation produces chemically active metabolic products and electrochemically active redox species that could interfere with the sensor performance. Hence, investigating the chemiresistive pH sensor in a complex biological medium would give some insight to use this material to develop sensitive conductometric micro sensors for micro bioreactor screening platforms.

Here we report our study on polyaniline functionalized Electrochemically reduced graphene oxide (ERGO-PA) based potentiometric and chemiresistive pH sensors for real time monitoring pH change in a *Lactococcus lactis* bacterial fermentation. *L. lactis* is a lactic acid producing bacterium, used in food fermentation and food preservative processes. It is mainly used in industry to produce cheese, yogurt and other dairy products. The lactic acid bacteria utilize sugar/carbohydrates and converts them into lactic acid through glycolysis. The bacteria use this process to generate energy necessary for its growth through ATP production. ATP is produced during the glycolysis by phosphorylation reaction in addition to the reduction of NAD^+ to NADH. The NAD^+ is recovered during the reduction of pyruvate to lactate by L-lactate dehydrogenase [2]. Lactic acid production is parallel to the bacterial growth in this fermentation process. As the concentration of acid builds up, the pH of the media decreases and finally the bacteria stop growing at a pH around 4. The pH is a crucial parameter in fermentation process, as it is indirectly used to monitor the bacterial growth and completion of fermentation process [3-4]. Hence, measurement of pH at every stage of the fermentation process is important.

The fermentation broth consists of a complex mixture of peptides, salts, carbohydrates and redox active molecules that have a tendency to interfere with the sensor surface [5-9]. Initially, our investigation in using the polyaniline functionalized electrochemically reduced

graphene oxide to measure, in real time, the pH in a *L. lactis* fermentation failed. Optimizing the manufacturing process of the sensor and adding a selective layer on top of the active sensing area of the electrode enabled us to use the electrode successfully in a fermentation process.

2. Materials and Experimental details

2.1. Graphite oxide preparation

Graphite oxide was prepared by Hummers method [6]. In a typical procedure, 3 g of graphite flakes (Sigma-Aldrich) was dispersed in 69 ml of H_2SO_4 (Merck), and 1.5 g of sodium nitrite (Sigma-Aldrich) was added to the suspension while stirring. The suspension was placed in an ice bath and continuously stirred followed by the slow addition of 9 g KMnO_4 (Sigma-Aldrich). Subsequently, 400 ml of distilled water was cautiously added to the mixture. The temperature immediately rose to 90°C and this temperature was maintained for 15 minutes. Afterwards, 7.5 ml of 30% H_2O_2 (Sigma-Aldrich) was added and the color of the suspension changed from brown to yellow. Finally, the graphite oxide suspension was washed several times with 5% HCl (Merck) and Milli Q water.

2.2. Electrochemically reduced graphene oxide (ERGO)

Graphene oxide (GO) stock solution was prepared by dispersing 10 mg of graphite oxide in 10 ml of Milli Q water and sonicated for 2 h in a sonication bath. 2 μl of GO solution was drop cast on a pre-cleaned Au electrode (Au disc electrode for potentiometric and interdigitated Au electrode for chemiresistive sensor) and the electrode was air dried for 24 h at room temperature. Electrochemical reduction of GO deposited Au electrode was carried out using cyclic voltammetry by scanning the electrode from 0 to -1.2 V with a scan rate of 50

mV/s in phosphate buffer at pH 7. Then the electrode was dried at 100°C in the oven for at least 12 hours.

2.3. Polyaniline functionalization on ERGO (ERGO-PA)

A thin film of polyaniline was functionalized on the surface of ERGO by electropolymerization of aniline using a concentration of 10 mM in 5 ml 1 M H₂SO₄. Cyclic voltammetry was used for the deposition and the electrode was cycled 50 times between 0 to 0.9 V at a scan rate of 50 mV/s.

After the polyaniline functionalization, the electrodes were coated with 5 µl of Nafion (5 wt %, Sigma-Aldrich).

2.4. Bacterial fermentation media preparation

L. lactis was grown in glucose M17 medium. M17 powder (9.3 g) (BD Difco™) was dissolved in 237 ml of Millipore and autoclaved at 121° C for 15 min. After cooling down to room temperature, 13 ml of 20% (w/v) autoclaved glucose solution was added.

2.5. Instruments and Methodology

The responses of the electrodes were measured by immersing the electrode in solutions with different pH values (pH 2.0 – pH 9.0). Britton and Robison universal buffer solution (0.04 M H₃PO₄, 0.04 M CH₃COOH, and 0.04 M H₃BO₃) was titrated with 0.2 N NaOH to adjust the pH to the desired value [4]. For potentiometric sensor, a 2 mm gold disc platinum wire, and Ag / AgCl were used as working, counter and reference electrode respectively (CH instruments, Austin, Texas, USA). The electrochemical measurements were performed with CH-Instruments (CH600 and CH760). A three-compartment electrochemical

cell was used for measurements. Potentiometric responses were obtained by measuring the open circuit potential (OCP) against a Ag/AgCl reference electrode, and the change in potential value was used as the sensor signal. For chemiresistor measurement Au interdigitated electrodes deposited on ceramic substrate, having four pair of electrodes (seven fingers) with 100 μm gap (interfinger spacing) between each electrode (BVT technology, Brno, Czech Republic) is used. For chemiresistive response, 100 mV constant voltage was applied between the source and drain, the resistance values are calculated from the output current and the change in resistance value was used as the sensor signal. FT-IR Tracer-100 (Shimadzu, Japan) was used for the FT-IR studies. The samples were scanned between 500 to 4000 cm^{-1} . Powder X ray diffraction (XRD) was performed with D8-Advance with LYNXEYE detector (Bruker, Germany). Perkin Elmer Raman station equipped with 785 nm laser was used for Raman measurements. Surface morphology was analyzed using NovaNanoSEM 650.

For continuous pH measurements, glucose M17 broth (5 ml) was pipetted into an autoclaved electrochemical cell, inoculated with *L. lactis* and incubated at 30°C. The solution was continuously stirred throughout the measurement for uniform mixing and to avoid sedimentation of bacteria at the bottom of the electrochemical cell. To measure the real time pH changes during the growth of *L. lactis*, the potential differences of the ERGO-PA were monitored for 72 hrs. The pH value before and after the growth of *L. lactis* was checked with a conventional glass electrode.

3. Results and discussion

3.1. Material and electrochemical characterization

A typical cyclic voltammetry of electrochemical reduction of GO (ERGO) and electropolymerization of aniline is shown in Figure 2. Cyclic voltammetry of GO showed an

intense reduction peak around -1.0 V with the onset potential of -0.8 V as described before [7-9]. The reduction of GO is an irreversible process which could be observed from the subsequent cycles. During the cycles the cathodic peak decreased completely, and no peak appeared in the anodic region (Fig. 1 (i)). Electropolymerization of aniline by cyclic voltammetry is shown in (Fig. 1(ii)), where the oxidation of aniline occurred at +0.7 V. The continuous increase of current density in the following cycles indicated the growth of a thin polyaniline film over the GO surface. The cyclic voltammetry of polyaniline deposition also showed redox peaks, which corresponded to the oxidation of reduced leucoemeraldine to emeraldine and its further oxidation to pernigraniline [10-12, 15].

The formation of graphite oxide and ERGO was confirmed by XRD, FT-IR and Raman spectroscopy (Fig. 2). XRD of graphite (Fig. 2.1) showed an intense peak at 26.5° which is related to hexagonal graphitic structures of (002) plane. Upon oxidation, the interfacing layer of graphite expanded and the peak shifted to a lower 2θ value of 10.7° , which indicates successful conversion of graphite into graphite oxide. The IR spectrum of GO (Fig 2 (i)) showed strong peaks at 1700 cm^{-1} , and 1010 cm^{-1} which are due to -C=O and -C-O stretching of the COOH and epoxide functional groups respectively. These peaks disappeared completely when GO was converted into ERGO indicating a complete reduction of the oxygen functional groups on the GO surface [13]. The Raman spectra of ERGO and ERGO-PA is presented in Fig. 2 (ii). The Raman spectrum of ERGO shows two intense bands at 1310 cm^{-1} and 1590 cm^{-1} , that correspond to the D and G bands of graphene sheets arising from defect sides and in plane vibrational mode of ERGO domains, respectively [14]. The high ratio of D to G band indicates that ERGO contains more defective sites due to the removal of the functional oxygen groups from GO. The Raman spectrum of ERGO-PA contains peaks at 1595 cm^{-1} , 1501 cm^{-1} , 1348 cm^{-1} , and 1168 cm^{-1} corresponding to quinoid and benzenoid ring stretching vibrations, C-N stretching vibration of protonated imine and

aromatic C-H in plane vibrations of polyaniline ring, respectively [15-19]. SEM images of ERGO-PA and ERGO-PA-NA are recorded and presented in (Fig. 3(i-iii)). SEM image of ERGO-PA shows that porous fiber network of polyaniline grown on the surface of ERGO. Surface of ERGO was completely covered and buried underneath the polyaniline fibers.

3.2. Potentiometric sensing of ERGO-PA-NA:

The pH response for ERGO and Nafion coated ERGO-PA electrode (ERGO-PA-NA) was tested by measuring potential differences from pH 2 to 9. The potential differences occur because of a different proton concentration according to the Nernst equation shown in Eqn.1.

$$E = E^{\circ} + [2.303RT / F] \log [H^{+}] \quad (1)$$

The ERGO-PA-NA electrode gave a near Nernstian response of 55 mV / pH with a linear regression coefficient of 0.998 (Fig. 3 (ii)) while ERGO without polyaniline gave poor sensitivity of 37 mV / pH (Fig. 3 (i)). The response time and stability of ERGO-PA-NA was obtained by continuously monitoring the open circuit potential over a period of time while the pH of the solution was increased by the addition of 0.2 N NaOH. The result is shown in Fig. 3 (iii). From the graph it can be seen that the ERGO-PA-NA electrode has a good stability with a response time of a few seconds.

3.2.1. Potentiometric continuous real time pH measurement in *L. lactis* fermentation:

For real time pH measurements in fermentation media, the ERGO-PA-NA electrode was directly immersed in the fermentation broth. The role of Nafion, a proton selective layer, was crucial to measure the pH in this experiment. As mentioned before, the fermentation medium contains a large number of chemicals that interfere with the pH measurement. The presence of a Nafion coating, acting as a cationic exchange membrane, prevented that these chemicals reached the surface of ERGO-PA. To confirm the role of Nafion, both the ERGO-PA and

ERGO-PA-NA electrodes were used to measure the pH in a growth experiment with *L. lactis*. Both electrodes showed a completely different behavior (Fig. 4). The potential values followed nicely the exponential growth curve of *L. lactis* in which there is a lag phase, an exponential phase, and a stationary phase. Lactic acid is produced during the exponential phase resulting in a decrease in pH. At the end of the growth, glucose is exhausted, and no further production of lactic acid takes place. In this stationary phase the pH remains constant. The accumulation of lactic acid changes the pH of the media, which was immediately sensed by the ERGO-PA-NA electrode and resulted in a change of open circuit potential. However, this was reversed in the case of ERGO-PA and this is most likely due to the interaction of negatively charged metabolic products with the ERGO-PA electrode surface.

To confirm that the increase in potential difference of the ERGO-PA-NA electrode was due to the growth of *L. lactis*, the optical density (OD) of the fermentation broth was measured at 600 nm (Fig. 5). The growth curve of *L. lactis* reflected the typical exponential curve of bacterial growth with the three distinct phases as mentioned before. The potential differences of the ERGO-PA-NA electrode followed the same trend as the growth curve, which confirmed that the potential difference of ERGO-PA-NA electrode was due to the growth and production of lactic acid. The pH of the fermentation broth was also measured with a conventional glass electrode at several time points. The pH correlated very well with the potential difference of the ERGO-PA-NA electrode measured at the same time. To obtain the exact pH value from the potential differences of ERGO-PA-NA the voltage values are converted to the pH using the Nernst equation. Not much difference was observed from the pH value obtained from the ERGO-PA-NA electrode and the pH value measured with a conventional glass electrode which indicated that the accuracy of the ERGO-PA-NA electrode in real time monitoring pH changes, is sufficient to be used in bacterial fermentations (Fig. 6).

3.3. Chemiresistive sensing:

For chemiresistive pH sensing, ERGO and polyaniline were deposited on an interdigitated gold electrode, and the same experimental procedures were followed as in the preparation of ERGO-PA. To protect the ERGO-PA surface 5 μ l of Nafion was drop cast on the ERGO-PA deposited electrode. For the chemiresistive pH sensing, the current response of the prepared ERGO-PA electrode and ERGO-PA-NA electrode was recorded separately in R-B buffer solution between pH 4-9. In Fig. 6 ((i) and (ii)) the resistance change was shown of ERGO-PA and ERGO-PA-NA electrode for pH 4-9. The resistance of both electrodes increased as the pH increased which is indicative of p-type semiconductive behaviour in which the holes are charge carriers [1, 20-22]. Changes of resistance, when exposed to different pH solutions, occur due to doping and de-doping of holes during protonation and deprotonation of polyaniline in ERGO-PA. As holes are charge carriers in a p type semiconductor, an increase in hole concentration during protonation increases the conductivity of ERGO-PA and a decrease in hole concentration during deprotonation lowers the conductivity. This behavior is clearly observed in Fig. 5. Both ERGO-PA and ERGO-PA-NA electrodes showed a sensitivity of 1.71 Ω / pH (Fig. 6 (iii)). The presence of Nafion, however, increased the resistance of the ERGO-PA-NA electrode (Fig. 6(ii)). The sensitivity of the ERGO-PA-NA electrode was similar to the ERGO-PA electrode which indicates that Nafion can be used to protect the surface of an ERGO-PA electrode without compromising the sensitivity of the electrode. From Fig. 6 (ii), it can also be seen that ERGO-PA-NA electrode not only shows good sensitivity but also good reversibility in the pH range 4-9. For real time pH measurement in *L. lactis*, ERGO-PA-NA electrode is dipped in the M17 medium and kept in there until the completion of the *L. lactis* growth. In order to follow the growth of the *L. lactis*, optical density measurement and the pH with a glass electrode of the culture was monitored externally by taking samples at selected intervals. In order to perform pH

measurement of ERGO-PA-NA electrode in *L. lactis* culture, the resistance of the electrode is measured and plotted against time (Fig. 8). The resistance of ERGO-PA-NA electrode is stable at 294.5 Ω initially and then started to decrease immediately after the beginning of *L. lactis* growth. The resistance of the ERGO-PA-NA electrode continuously changed and reached a stable value at 288.6 Ω at the point when the growth of *L. lactis* and production of lactic acid stopped. At the end of the growth the pH of the medium changed from 7.2 to 4.8 corresponds to 2.4 pH unit and the resistance of ERGO-PA-NA changes from 294.5 Ω to 288.6 Ω which is corresponds to change of 5.9 Ω . This change of resistance value with respect to the change of pH of the media is well within the expected pH range measured in R-B buffer solution (inset Fig. 8). This behaviour confirms that resistance of the ERGO-PA-NA electrode is sensitive to the pH change of M17 medium due to acidification by the production of Lactic acid during the *L. lactis* growth and can be used as a referenceless micro pH sensor in micro bioreactor screening platforms. The SEM image of the ERGO-PA-NA electrode (Fig. 3(iii)) was recorded after the measurement in *L. lactis* fermentation. The SEM image displays a uniform Nafion coating completely covering the ERGO-PA surface, even after long time (>50 hrs) in the fermentation medium.

4. Conclusions

Polyaniline functionalized electrochemically reduced graphene oxide based chemiresistive pH sensor is studied for real time monitoring the pH in a *L. lactis* fermentation medium. Graphene and polyaniline were prepared by electrochemical reduction of GO and electropolymerization of aniline using cyclic voltammetry. The formation of ERGO-PA was confirmed by IR and Raman spectroscopy. Nafion coated ERGO-PA electrode is successfully used as potentiometric and chemiresistive pH sensor in *L. lactis* fermentation for real time monitoring pH changes. Nafion prevented the negative interaction of metabolic products with

the ERGO-PA. Our results show that a Nafion coated ERGO-PA electrode can be used as a micro pH sensor for real time monitoring pH changes in complex biological processes.

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6. Figure captions:

Fig. 1: Cyclic voltammetry of GO reduction (i) and polyaniline deposition (ii) at a scan rate of 50 mV/s

Fig. 2: XRD of Graphite and Graphite oxide (i) FT-IR spectrum (ii) of GO (a) and ERGO (b), and the Raman spectrum (iii) of ERGO (a) and ERGO-polyaniline (b)

Fig. 3: SEM images of ERGO-PA (i-ii) with different magnifications and ERGO-PA-NA (after the measurement for 50 hrs in buffer and fermentation medium) (iii)

Fig. 4: pH sensitivity of ERGO (i), ERGO-PA-NA (ii) and stability of ERGO-PA-NA (iii)

Fig. 5: Continuous potential measurements of ERGO-PA-NA (i) and ERGO-PA (ii) during the growth of *L. lactis*

Fig. 6: Relation between the optical density (OD), pH measured with a conventional glass electrode and the pH value obtained with an ERGO-PA-NA electrode during growth of *L. lactis* (i).

Fig. 7: Resistance change of ERGO-PA (i) and ERGO-PA-NA (ii) against time from pH 4 to 9 and resistance value of ERGO-PA-NA at different pH (iii).

Fig. 8: Real time continuous resistive change of ERGO-PA-NA in *L. Lactis* fermentation process, inset shows expected resistance change of ERGO-PA-NA for pH 4-7 measured in R-B buffer solution.

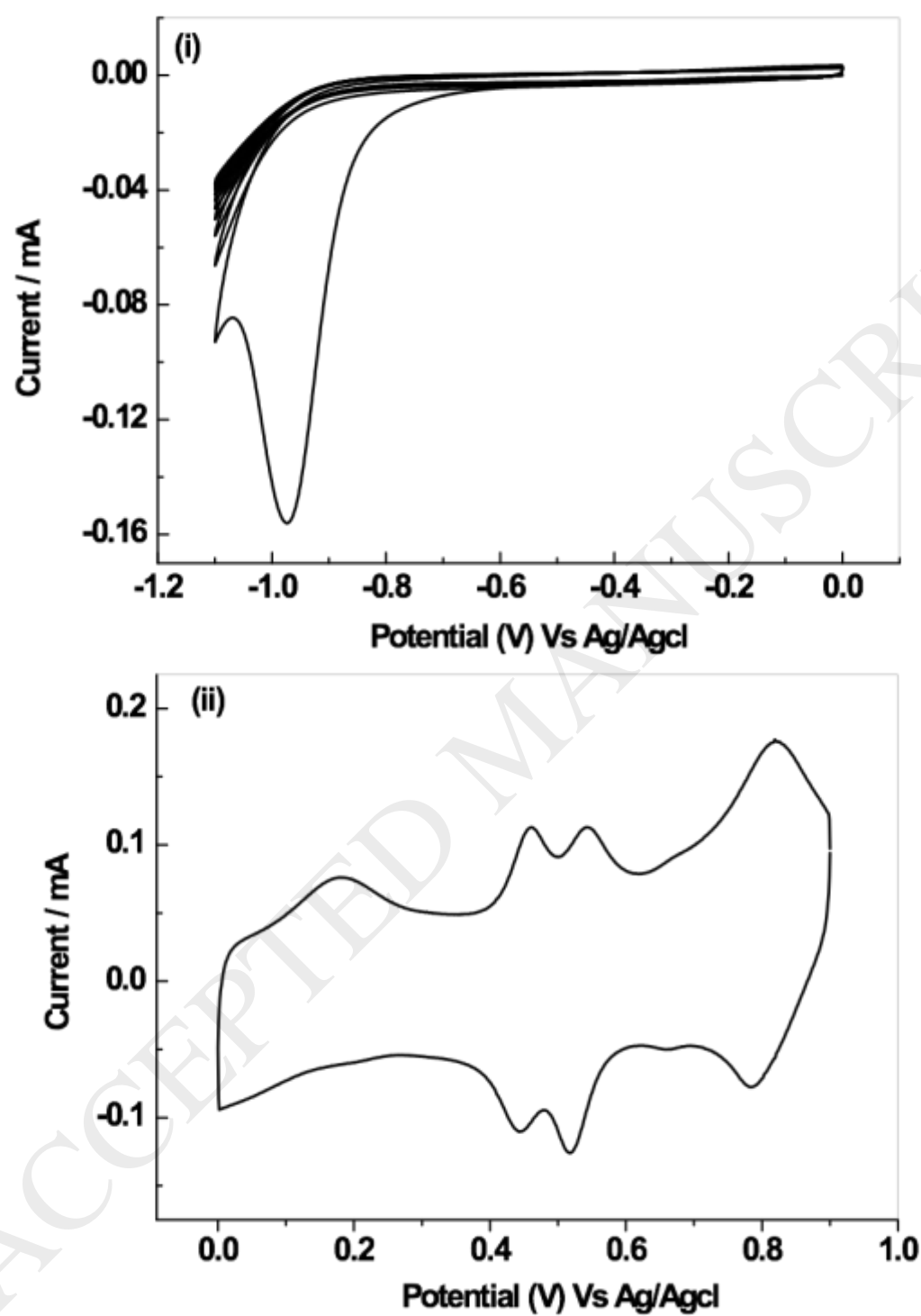


Fig.1

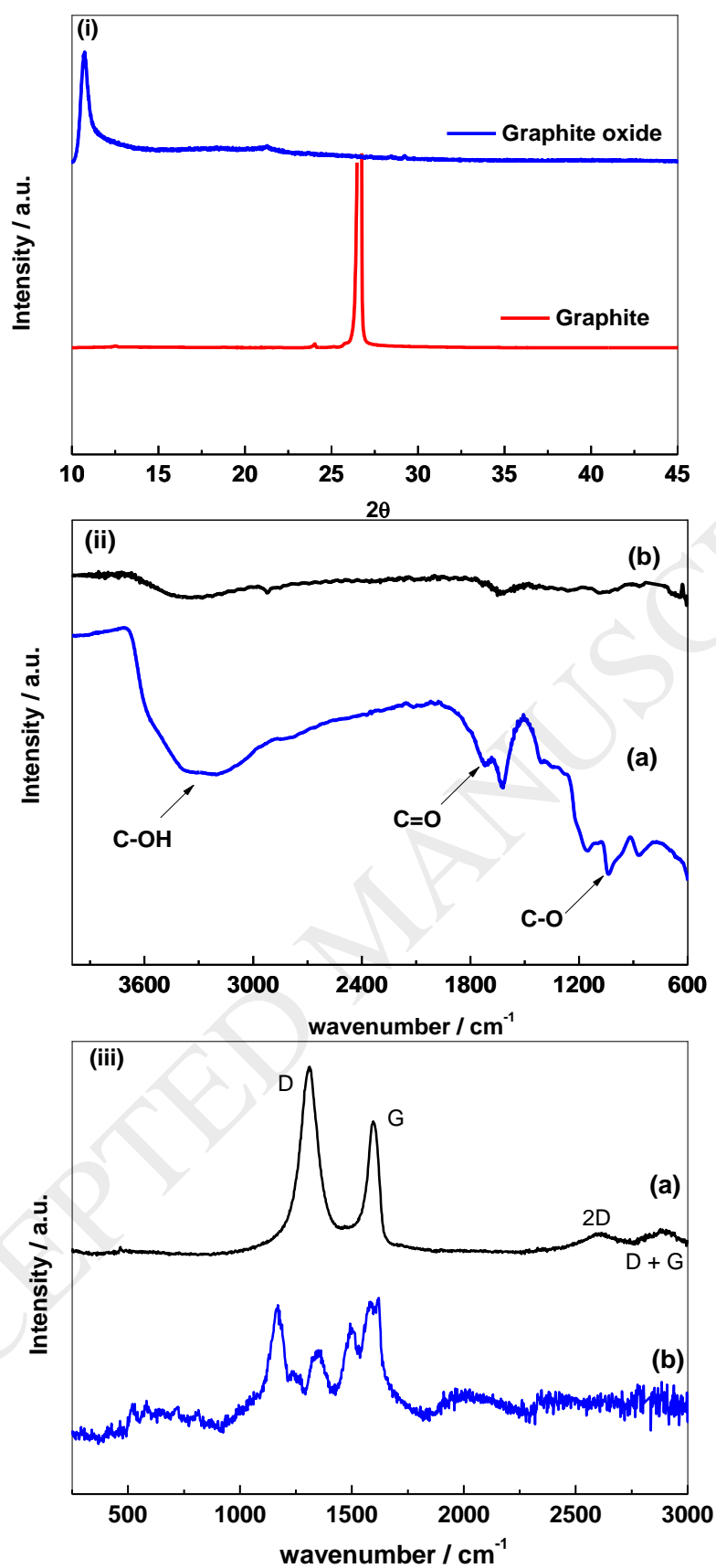
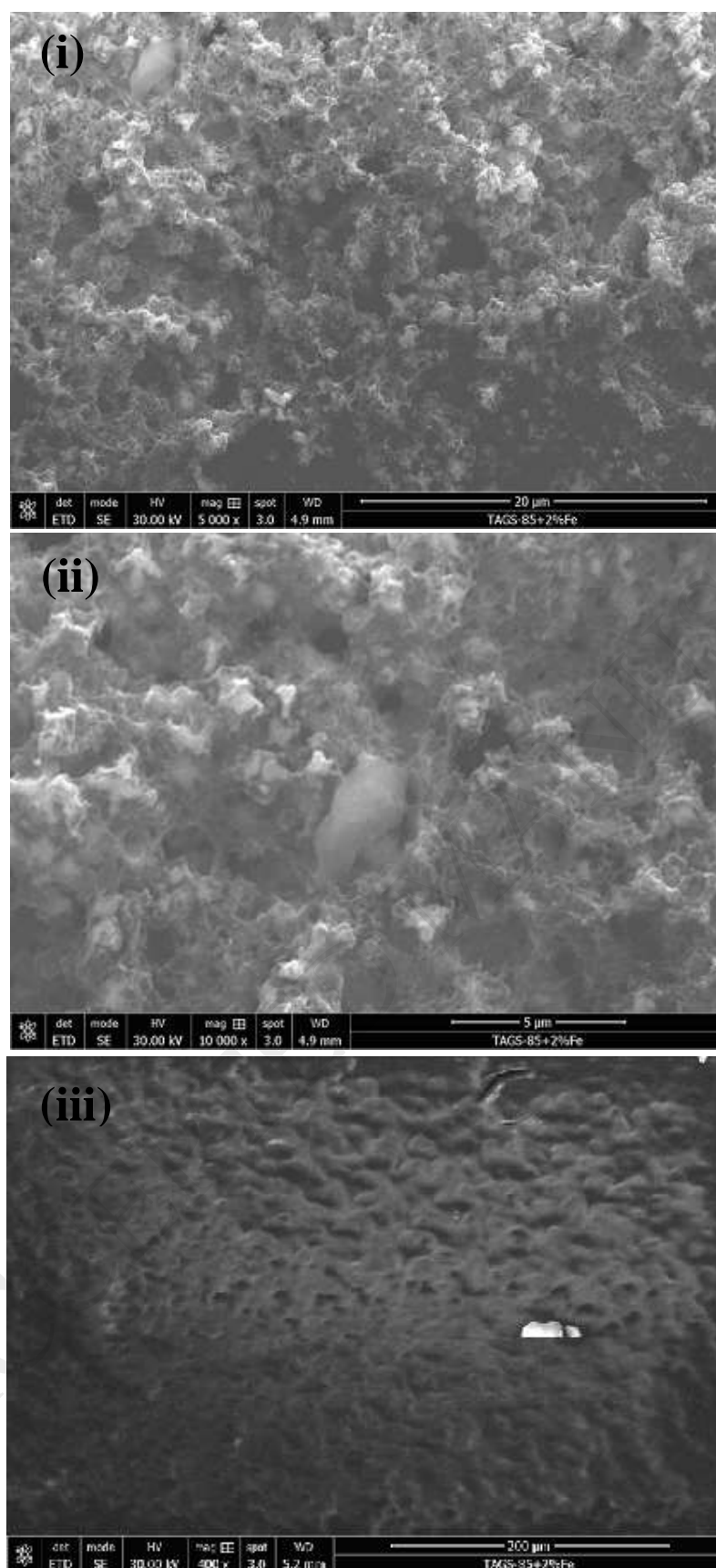


Fig. 2

**Fig. 3**

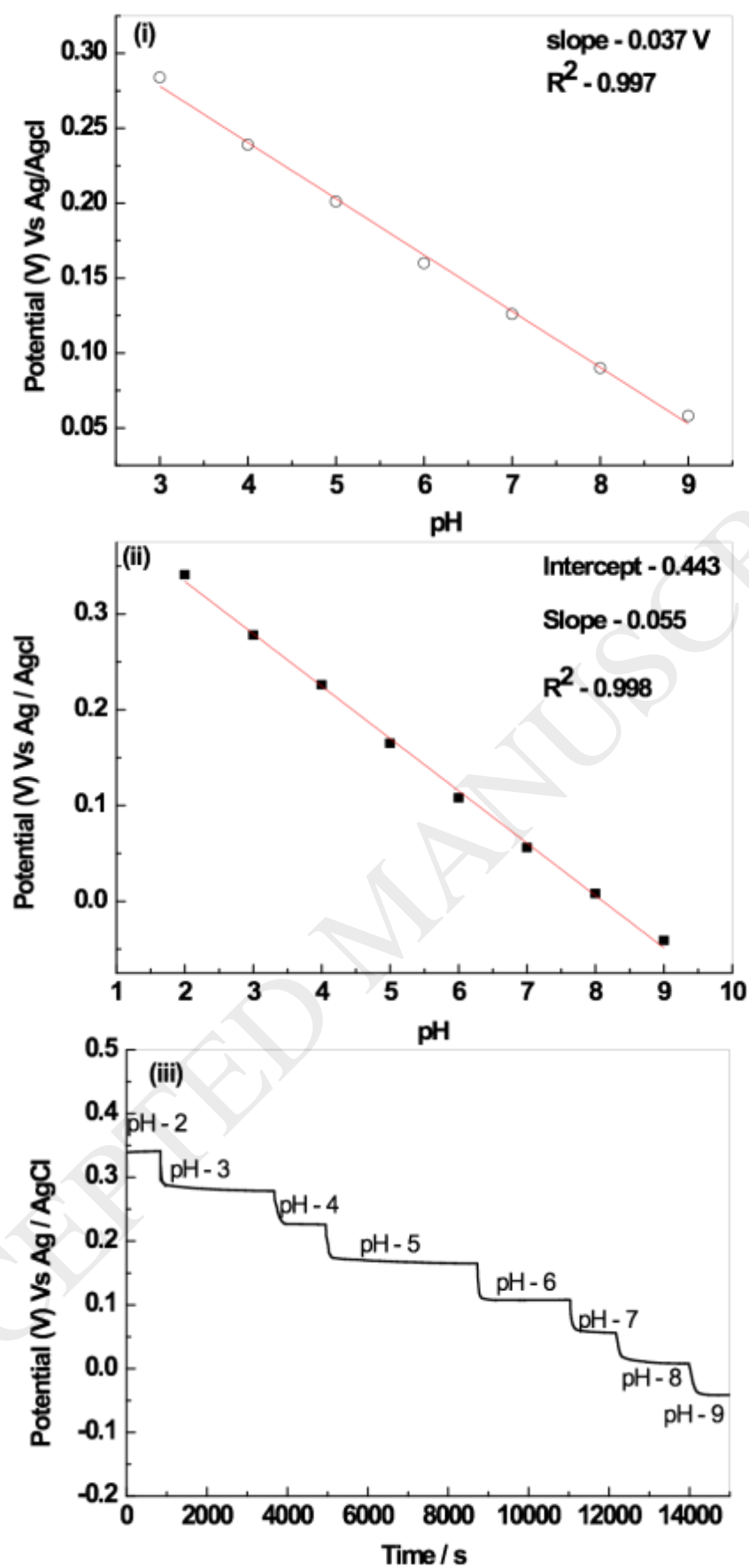


Fig. 4

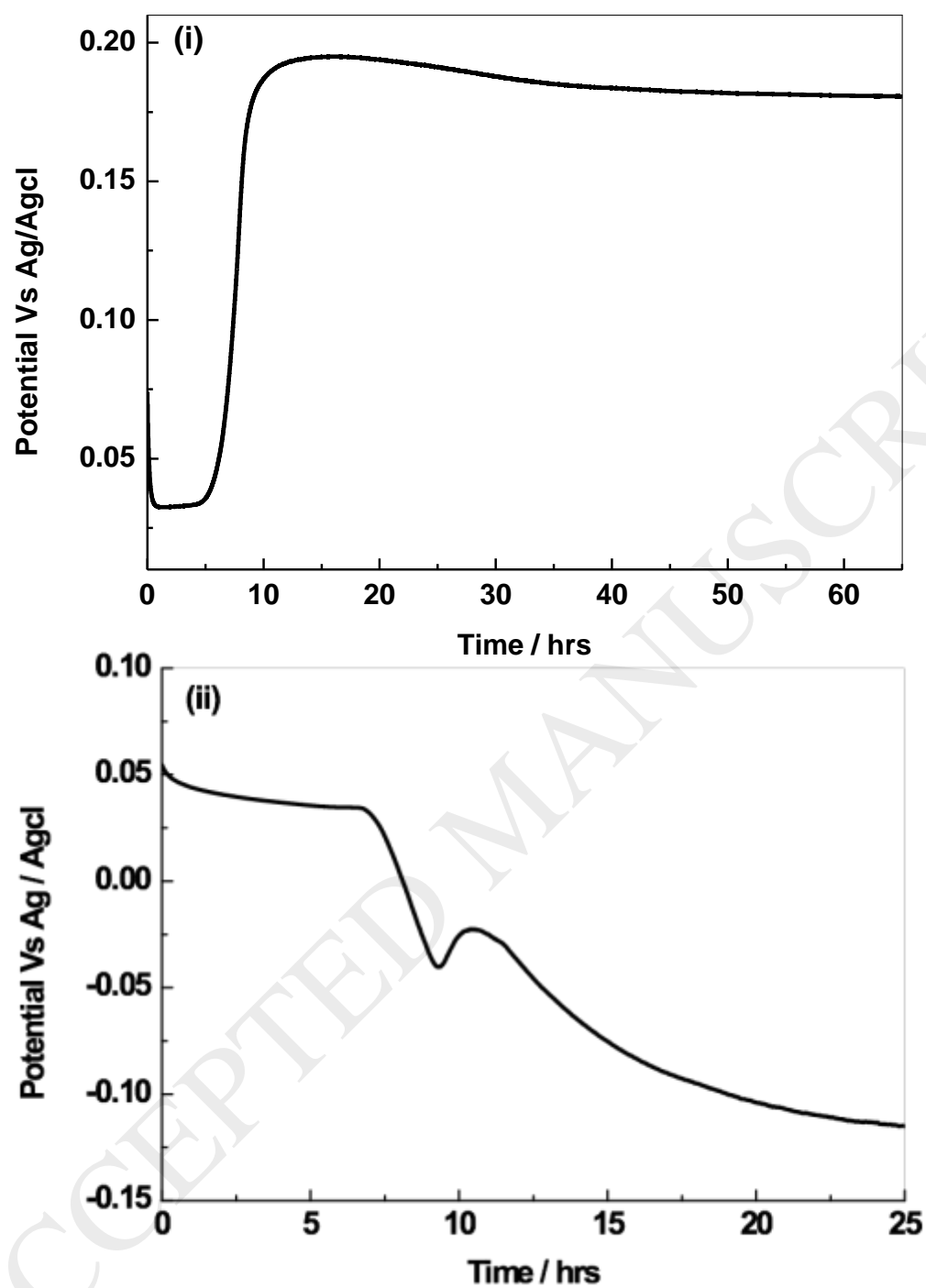


Fig. 5

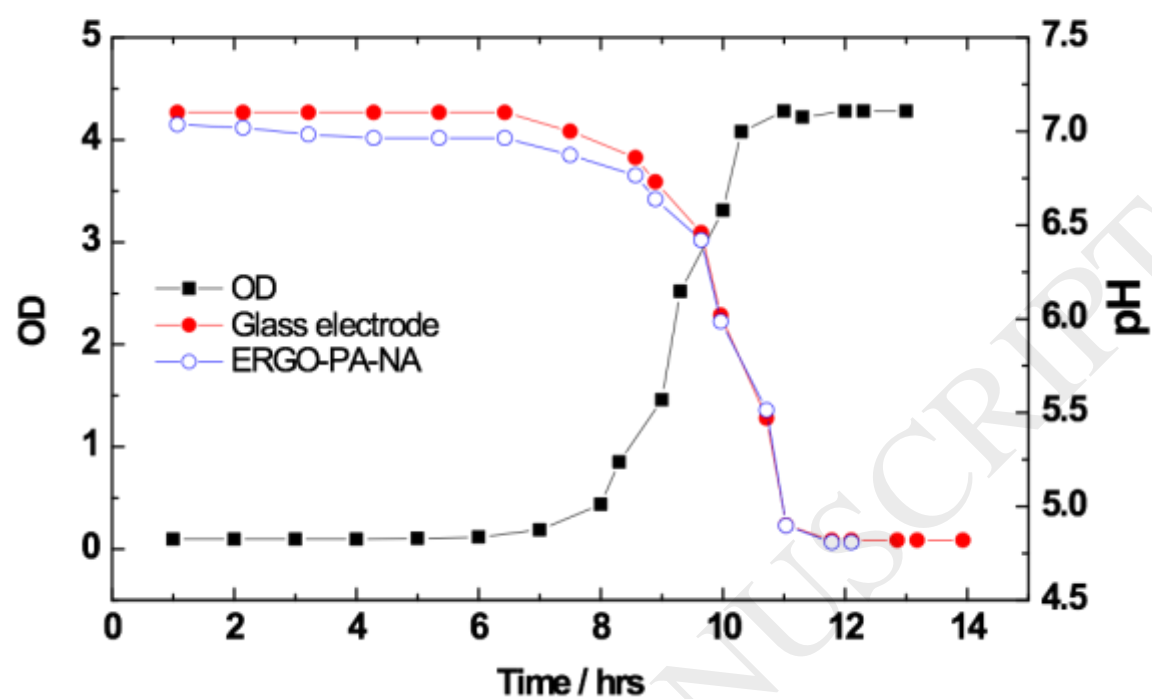


Fig. 6

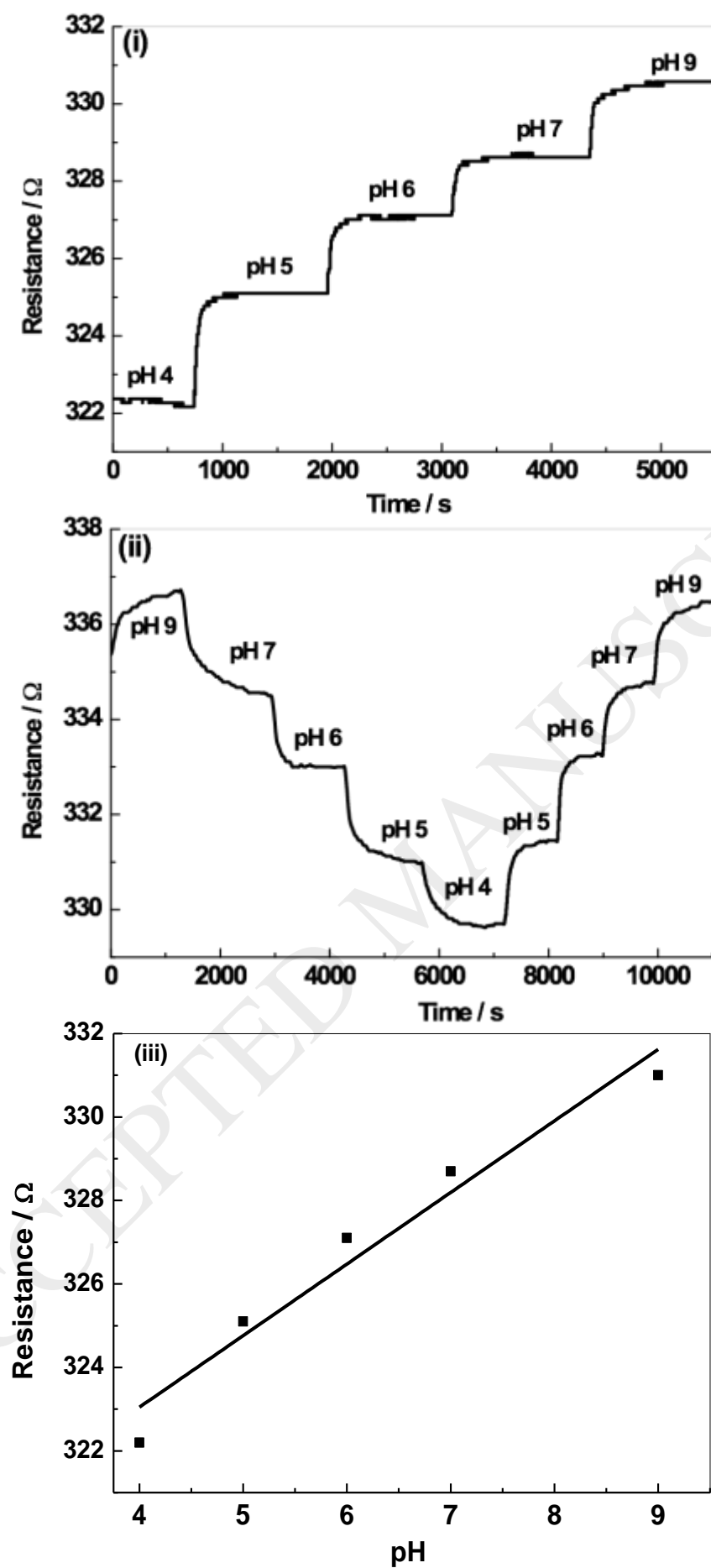


Fig. 7

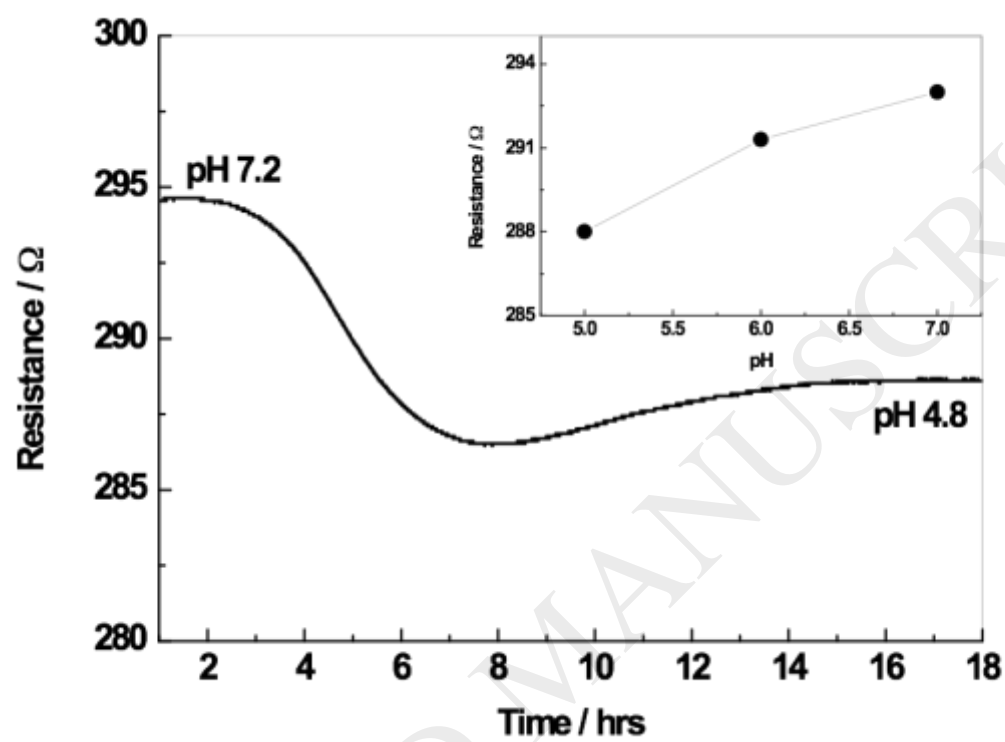


Fig. 8

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Biographies

Prof. Gert Jan Willem Euverink (1966) finished his PhD in microbiology/ biochemistry in 1995 at the University of Groningen, The Netherlands. From October 1994 until May 1998 he was a post-doctoral researcher at the Dutch Institute for Carbohydrate research (NIKO-TNO) where he discovered a new enzymatic derivative of starch. In 1998 he started a high-throughput screening laboratory in the department of Microbial Physiology at the University of Groningen and applied robot technology for the screening of new biocatalysts. In 2004 he became deputy scientific director of Wetsus, Centre for sustainable Water Technology in Leeuwarden. Since November 2011 he has been working as professor in university of Groningen and he is involved in the supervision of master students, PhD students and post-docs.

Mr. Selvaraj Chinnathambi received master degree in chemistry (2009) from Thiagarajar college affiliated with Madurai Kamarajar university. Before joining university of Groningen for phd degree in 2014, he worked as a project assistant in Indian Institute of Science, Bangalore, where he worked on material development for Lithium batteries and supercapacitors. Now he is working on developing referenceless micro sensor arrays for multi micro reactor screening platforms .

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