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Development of a Temperature-pulse Enhanced Electrochemical Glucose Biosensor and Characterization of its Stability via Scanning Electrochemical Microscopy

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Abstract: Glucose oxidase (GOx) is an enzyme frequently used in glucose biosensors. As increased temperatures can enhance the performance of electrochemical sensors, we investigated the impact of temperature pulses on GOx that was drop-coated on flattened Pt microwires. The wires were heated by an alternating current. The sensitivity towards glucose and the temperature stability of GOx was investigated by amperometry. An up to 22-fold increase of sensitivity was observed. Spatially resolved enzyme activity changes were investigated via scanning electrochemical microscopy. The application of short (<100 ms) heat pulses was associated with less thermal inactivation of the immobilized GOx than long-term heating.

Keywords: Biosensors · Electrochemistry · Thermoelectrochemistry · Scanning electrochemical microscopy

1 Introduction

Scanning electrochemical microscopy (SECM) is a scanning probe technique frequently used for the electrochemical characterization of surfaces and interfaces. Common application fields include corrosion research [1], biological studies with cells [2], and enzymes [3], as well as material characterization [4]. Among the various measuring modes of SECM, the generation/collection (G/C) mode suffers from limited reproducibility and contrast issues due to a growing diffusion layer at the generator electrode. Forced convection was introduced into the measurement cells by different means [5], resulting in increased currents and reproducibility of images as stable diffusion layers can be established at the generator electrode. Furthermore, this also led to contrast improvement in feedback mode imaging of SECM due to an enhanced feedback cycle at conductive surfaces [6].

As elevated temperatures increase the molecular motion within solutions as well, another option to benefit from the effects of forced convection is employing heated substrates in SECM. In a recent effort, our group presented a setup for the implementation of flattened Pt microwires as substrates that are heated by an alternating current (AC) according to the concept of "hot-wire electrochemistry" [7]. The latter originates from the work of Gründler and co-workers [8,9], featuring a thin (<25 µm diameter) gold or platinum microwire employed as working electrode (WE), heated by an AC with a frequency ≥100 kHz. In addition to the AC heating circuit, voltammetric measurements can be performed simultaneously. So far, hot-wire electrochemistry was utilized in various studies, including the trace analysis of various elements [10], or the behaviour of DNA and RNA under temperature influence [11]. Moreover, it is the basis for techniques like temperature pulse voltammetry [12], enabling even the analysis of substances above the boiling point of the electrolyte, as short temperature pulses do not lead to boiling effects. Studies concerned with temperature and its effects on SECM have shown that higher temperatures increase the contrast between conductive and non-conductive surfaces [13]. To make use of this effect, Boika et al. have developed a setup termed "hot tip SECM", integrating the technology behind hot-wire electrochemistry into the SECM probe resulting in increased thermal convection in the vicinity of the probe tip [14].

Electrochemistry in general is a useful tool for the analysis of enzymes, as their activity can be correlated to the electrochemical current originating from the consumption or generation of electroactive products. This principle led to the development of numerous biosensors [15], such as glucose biosensors using glucose dehydrogenase or glucose oxidase (GOx) to determine glucose concentrations. In order to enhance the performance

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characteristics of biosensors, efforts were made to increase enzyme stability and activity by immobilization techniques [16], as they may affect optimal pH and temperature region of enzymes. In case of GOx, efforts were made to immobilize the enzyme and increase its stability at high temperatures. T.-F. Tseng et al. have immobilized GOx in a Nafion matrix [17], leading to increased stability and activity at high temperatures generated in the context of hot-wire electrochemistry. Especially, temperature pulses of 30 s duration showed a significant increase in GOx activity. Other groups have presented electrochemical GOx biosensors as well, e.g. using porous silica [18] or silica mesocellular foams [19] for immobilization.

For the in-depth analysis of enzymatic biosensors, SECM can be applied, especially as a tool in the development of miniaturized systems [20]. Generally, the G/C modes and the redox competition mode enable the investigation of the kinetics at catalytically active sites of GOx substrates. In the G/C mode, a product from an enzymatic reaction can be collected at the SECM probe, while in the redox competition mode, the probe is set to a potential where it is competing with the enzyme in terms of educt consumption. Both modes have been applied for the characterization of GOx-based sensors [21]. A mediator-free technique measuring the impedance was reported as well [22].

In this work, the aim was to study the different impact of long-term vs. pulsed heating on the activity of GOx. We immobilized GOx on the surface of a flattened Pt microwire using a commercial enzyme spotter. The wire could be heated according to the hot-wire concept, leading to a temperature-adjustable glucose biosensor at which short temperature pulses were applied. The sensitivity of the sensor was studied for different glucose concentrations and pulse temperatures. A spatially resolved study of thermal deactivation of the enzyme caused by long-term heating and short temperature pulses was investigated using SECM. Images recorded in G/C mode before and after heating were used to reveal changes in GOx activity.

2 Experimental

2.1 Chemicals

All solutions were prepared using Milli-Q water (Milli-Q Advantage A10 system, Merck Millipore, Darmstadt, Germany). An aqueous 5/5 mM mixture of ferro/ferricyanide ($K_3Fe(CN)_6/K_4Fe(CN)_6$, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was used for temperature calibration and substrate characterization measurements. The following solutions were all prepared with phosphate buffer (pH 7) if not stated otherwise. The phosphate buffer was prepared using sodium dihydrogen phosphate monohydrate (Merck KGaA, Darmstadt, Germany) and di-sodium hydrogen phosphate dodecahydrate (Merck KGaA, Darmstadt, Germany). Feedback mode SECM imaging was carried out with 1.5 mM ferrocenemethanol (FcMeOH, 99 %, ABCR, Karlsruhe, Germany). G/C mode SECM imaging was performed with 10 mM glucose (Merck KGaA, Darmstadt, Germany). A buffered (pH 7,4) GOx solution with 4 U/mL (Type X-S, 100,000–250,000 units/g, Merck KGaA, Darmstadt, Germany) was used for drop-coating.

2.2 Materials

A detailed description of the preparation of heated wire substrates for SECM studies is given elsewhere [7]. Pt wires of 25 µm diameter (Goodfellow GmbH, Hamburg, Germany) were used for the preparation of heatable electrodes used as the substrates and for the fabrication of ultramicroelectrodes (UMEs) as probes for SECM imaging. For fixation of the flattened Pt wire epoxy resin (Epoxydharz L, R&G Faserverbundstoffe GmbH, Waidenbuch, Germany) was used. Microscope slides served as base for the substrates. Copper wires (0.5 mm inner diameter, 1.1 mm outer diameter, Pollin Electronic GmbH, Pförring, Germany) were soldered to the Pt wire to establish electrical contact to the substrates. Soldering joints were sealed by applying hot glue (Pattex, Henkel AG & Co. KGaA, Düsseldorf, Germany). The substrate used in this work had a resistance of 5.1 Ω.

For electrochemical measurements, a Pt wire of 0.5 mm diameter was used as counter electrode together with a Ag/AgCl 3 M KCl reference electrode. The potentials listed herein are referring to this system.

2.3 Instruments

All experiments were carried out with a SECM 920C (CH Instruments, Austin, Texas, USA) equipped with a 25 µm diameter Pt UME with an RG value (ratio between total tip diameter and platinum disk diameter) of 2, which was fabricated according to a procedure described elsewhere [23]. Heating during temperature calibration was realized by applying an external current with a direct current power source (Voltcraft HPS-13015, Conrad Electronic AG, Wollerau, Switzerland) to Peltier elements (KIMILAR, TEC1-12706), which were attached to the measurement cell. Drop-coating of GOx was performed with a sciFlexArrayer S3 (Scienion, Berlin, Germany). Heating of substrates was achieved with a laboratory-built AC source already used in a previous study [7] and fabricated according to [24]. The AC source consisted of a radio frequency generator and a transformer, yielding a 100 kHz AC. The special electronic configuration [24] allowed the simultaneous recording of amperometric signals.

2.4 Temperature Calibration

In order to apply heating of defined temperature, a temperature calibration was carried out with a 25 µm diameter UME as working electrode in a solution of

5/5 mM ferro/ferricyanide and 1 M KCl. The electrochemical cell was held at constant temperatures of 15.6, 20.7, 29.0, 35.3, 46.3, and 55.4 °C. When the temperature remained constant for 1 min, the open circuit potential (OCP) was measured for a duration of 200 s with a sampling rate of 0.1 s, and the values during the last 100 s were averaged. To prevent a temperature-induced potential shift of the reference electrode potential, the reference electrode was connected to the measurement cell via a capillary-based salt bridge. This method for temperature determination was previously applied in other studies and was found to be applicable for short temperature pulses [8,12]. It has to be noted that temperature determinations based on OCP measurements show limited accuracy in case of very short heating pulses due to the kinetics of OCP equilibration. However, in the present case it is considered as an appropriate method for temperature determinations [8,12]. Furthermore, only the temperature of the wire surface can be determined but not the temperature of the surroundings (within the enzyme layer), making overall temperature estimations in all relevant regions of the sensing device impossible.

2.5 Pulse Characterization

All of the following experiments were executed with the setup illustrated in Figure 1A. The UME for localized activity investigations was connected to working electrode channel 1 (WE1). Working electrode channel 2 (WE2) was used to record the amperometric current at the substrate electrode. In addition, the substrate (separately shown in Figure 1B) was connected to the AC generator

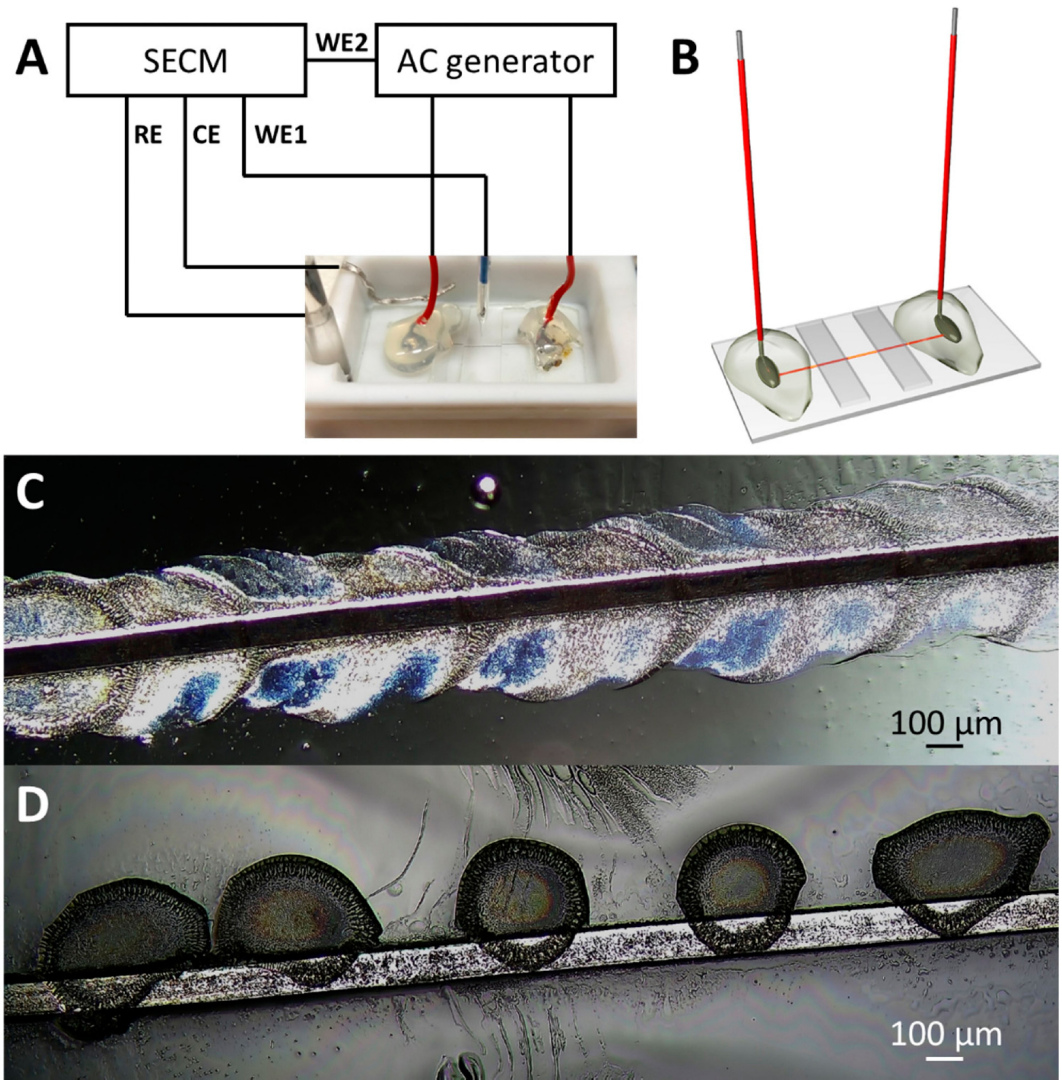

Fig. 1. A. Schematic experimental setup. Working electrode 1 channel (WE1) of the SECM was used for probe control. Working electrode 2 channel (WE2) was connected to the heatable substrate with an AC generator interconnected to enable AC heating. B: Illustration of the substrate with a flattened Pt wire as heatable electrode. Two additional glass slides were placed on top of the wire for means of mechanical stabilization (grey stripes). C/D: Micrographs of glucose oxidase covering the entire wire of the substrate (C) and applied as spots via drop-coating (D).

for in situ heating. The electrochemical setup was completed with counter and reference electrodes.

For the determination of the temperatures reached during heat pulses, the OCP was measured over time while applying 1/5/10/50/100 ms heat pulses with an idle time of 5 s after every pulse. Starting from 30 mA, the heating current was increased by 30 mA with every pulse, up to a magnitude of 600 mA. Data was acquired with a sampling rate of 1 ms. This procedure was performed three times for each pulse duration, and the shift of OCP was converted to temperature values according to the temperature calibration. A solution of 5/5 mM ferro/ferricyanide and 1 M KCl was used for this set of experiments.

To optimize the idle time between pulses applied in later experiments, the impact of idle time on the amperometric response was investigated. The same pulse sequence as before was applied to the substrate with idle times of 3, 5 and 8 s while performing chronoamperometry. A potential of 0.5 V was applied to induce mediator oxidation.

2.6 Temperature-pulsed GOx Biosensor

As GOx is frequently used for sensing glucose in biosensors, we investigated the impact of pulsed heating on the electrochemical response and the stability of the enzyme. In a first set of experiments, 5 µL of GOx solution were dispersed over the substrate, ensuring that the entire wire is covered with solution as shown in Figure 1C. After the solution dried, the substrate was placed in the electrochemical cell filled with 5 mL of phosphate buffer (pH 7). Chronoamperometry with a substrate potential of 0.8 V was then conducted while the glucose concentration inside the cell was raised stepwise. The concentration was increased by steps of 0.1 mM by the addition of 50 µL of 1 M glucose, up to a final concentration of 1.0 mM to cover a concentration range suitable for fundamental studies. After each addition, the solution was mixed, and as soon as a steady-state current was observed, 7 heat pulses of 100 ms duration with 8 s idle time were applied to the substrate. The experiment was repeated with different pulse temperatures, applying pulses that led to wire temperatures of 24, 34, 53, 65, 72, 79, and 106 °C. For each temperature, the GOx coating was renewed by cleaning the substrate with isopropanol and water, followed by reapplication of GOx solution when the substrate had dried.

Furthermore, the impact of long-term heating vs. short temperature pulses on the local GOx activity was studied. For these experiments, GOx was applied to the wire by drop-coating, resulting in a pattern as shown in Figure 1D. For each GOx spot, 20 drops with a drop volume of 319 pL were applied. The localized activity at these GOx spots was investigated by recording SECM images in feedback and G/C modes. In an area containing both bare wire and enzyme spot, SECM images covering 400× 500 µm with a pixel size of 10 µm and a scan rate of 50 µm s^{-1} were recorded. Feedback mode imaging in 1.5 mM FcMeOH was performed to locate the position of the Pt wire, while in G/C mode imaging, formed H_2O_2 in 10 mM Glucose indicated the position and activity of GOx spots. When switching measurement modes, the cell was flushed 3 times with phosphate buffer before adding the new solution. In G/C mode, the H_2O_2 generated by GOx during glucose conversion was oxidized at the SECM tip at a potential of 0.8 V as an indicator for GOx activity. Afterwards, the wire was either heated for 30 s at 42 °C or a short heat pulse (100 ms, 106 °C) was applied. To investigate localized activity changes, another G/C SECM image was recorded with the same parameters as before.

3 Results and Discussion

3.1 Pulse Characterization

The temperature reached during 100 ms heat pulses was investigated by measuring the OCP over time due to its temperature dependence. Figure 2A shows the OCP measured at an uncoated substrate for the application of 20 heat pulses of 30 to 600 mA heat current. The heat intensity increased by 30 mA with every pulse. When no heat was applied, an OCP of 0.285 V was measured. Heat pulses of 30 mA resulted in a negligible change in OCP. With elevating pulse intensity, higher OCP differences were observed, increasing exponentially up to a heating current of 300 mA. After that, a linear increase of the OCP shift was observed, as well as a slight baseline shift towards lower potentials. The latter was assumed to happen due to the glass around the wire being heated by the pulses, keeping the wire slightly above the initial temperature between the pulses.

In Figure 2B, the OCP differences from 3 measurements were averaged and converted to temperature values according to the temperature calibration given in Figure S1 in the supporting information (SI). The pulses led to reproducible temperatures when heating currents of up to 360 mA were applied. For stronger pulses, standard deviations of up to ±4 °C (n = 3) were obtained. For the highest heating intensity of 600 mA, a temperature of 106 °C could be reached. Temperatures beyond the boiling point are possible since the bubble formation associated with the boiling process is kinetically delayed. Even higher temperatures should be possible, as temperatures up to 250 °C were reported [25]. We also studied the temperatures reached with shorter heat pulses of 1, 5, 10, and 50 ms duration. The results are shown in Figure S2 in the SI. A similar trend in temperature increase was observed, but shorter pulses resulted in lower temperatures. For very short pulses of 1 ms, the temperature determination was inaccurate as the sampling rate during OCP measurements was 1 ms. Nevertheless, we decided to apply 100 ms pulses in the forthcoming experiments in order to reach the highest possible temperature.

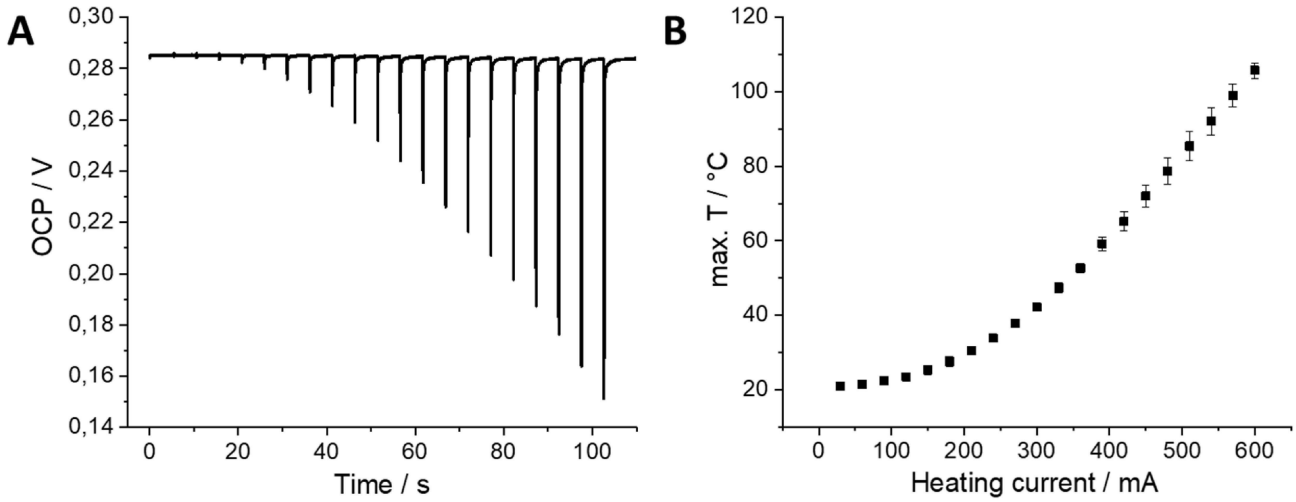

Fig. 2. A. Open circuit potential measurement over time while applying 100 ms heat pulses of increasing intensity to the substrate. Heating current increased by 30 mA steps from 30 to 600 mA. The time between each pulse was 5 s. B: Corresponding temperature for every heat pulse applied. The temperature was determined 3 times for all heating intensities.

Furthermore, to optimize the idle time for the upcoming amperometric experiments, pulses of increasing intensity with idle times of 3, 5 and 8 s were applied while performing chronoamperometry. Short idle times led to an increased current between pulses, as shown in Figure S3 in the SI. Therefore, an idle time of 8 s was chosen for the next measurements.

3.2 Temperature-pulsed Biosensor

The impact of temperature pulses on the activity of GOx used in a biosensor was investigated. The amperometric current from the oxidation of H_2O_2 was measured at different glucose concentrations in addition to temperature pulses applied for every glucose concentration. Figure 3A–C show chronoamperometric experiments where the glucose concentration was increased by increments of 0.1 mM from 0.1 to 1.0 mM and heat pulses were applied to the substrate leading to short increases of the wire temperature to 24 (A), 65 (B) and 106 °C (C). After each glucose addition, the solution was mixed (visible as a noisy current increase) and 7 heat pulses were applied when a constant current had established.

In the first 4–5 sets of peaks, artifacts frequently occurred, leading to the current peaking in both positive and negative direction. The origin of this effect is unknown but happens to disappear at concentrations higher than 0.5 mM. At glucose concentrations $\geq$0.5 mM, the increase of current appeared to be linear for all temperatures investigated as shown in Figure S4 in the SI. In Figure 3A, the temperature pulses led to a temperature increase from 20 °C to 24 °C. This resulted in small current increases ranging from 50 to 200 nA, with stronger increases observable at higher glucose concentrations. At 65 °C in Figure 3B, the current increase was significantly higher compared to Figure 3A, with a current increase between 0.5 to 3 µA, proportional to the glucose concentration. With peak temperatures of 106 °C (Figure 3C), the current during the very first pulse was initially very high at 10 µA but continued to decrease down to 3 µA for the last pulse at 0.2 mM glucose concentration. Afterwards, the peak current increased with glucose concentration up to a value of 7.5 µA. The initial decrease might have been caused by enzyme degradation in close vicinity to the heated wire due to the very high temperature, but a certain amount of GOx remained active since the current kept increasing over time with the glucose additions. It should be noted that the increased currents measured are the result of both increased GOx activity and enhanced conversion of H_2O_2 at the electrode, both resulting from higher temperatures. The single effects were previously investigated in a study carried out by T.-F. Tseng et al. [17], where a similar set of experiments was conducted, but an additional chronoamperometric measurement in H_2O_2 solution was performed to separately study the effect of temperature on the H_2O_2 conversion at the electrode.

To determine the increase of sensitivity, the ratio between peak current and the current measured 0.5 s prior to the heat pulse was used. Due to the linear increase of response, pulses applied at glucose concentrations between 0.5 and 1.0 mM were considered for the sensitivity determination. The ratios, termed "sensitivity increase factor" herein, for all investigated temperatures are given in Figure 3D. The remaining chronoamperometric measurements for temperatures which are not included in Figure 3A–C are depicted in Figure S5 in the SI (34, 53, 72, and 79 °C). Generally, with stronger heat pulses, a higher sensitivity could be achieved. Up to temperatures of 79 °C, the sensitivity remained highly reproducible with an increase factor of up to 10. For 106 °C, the current response increased 22-fold but with a high uncertainty of

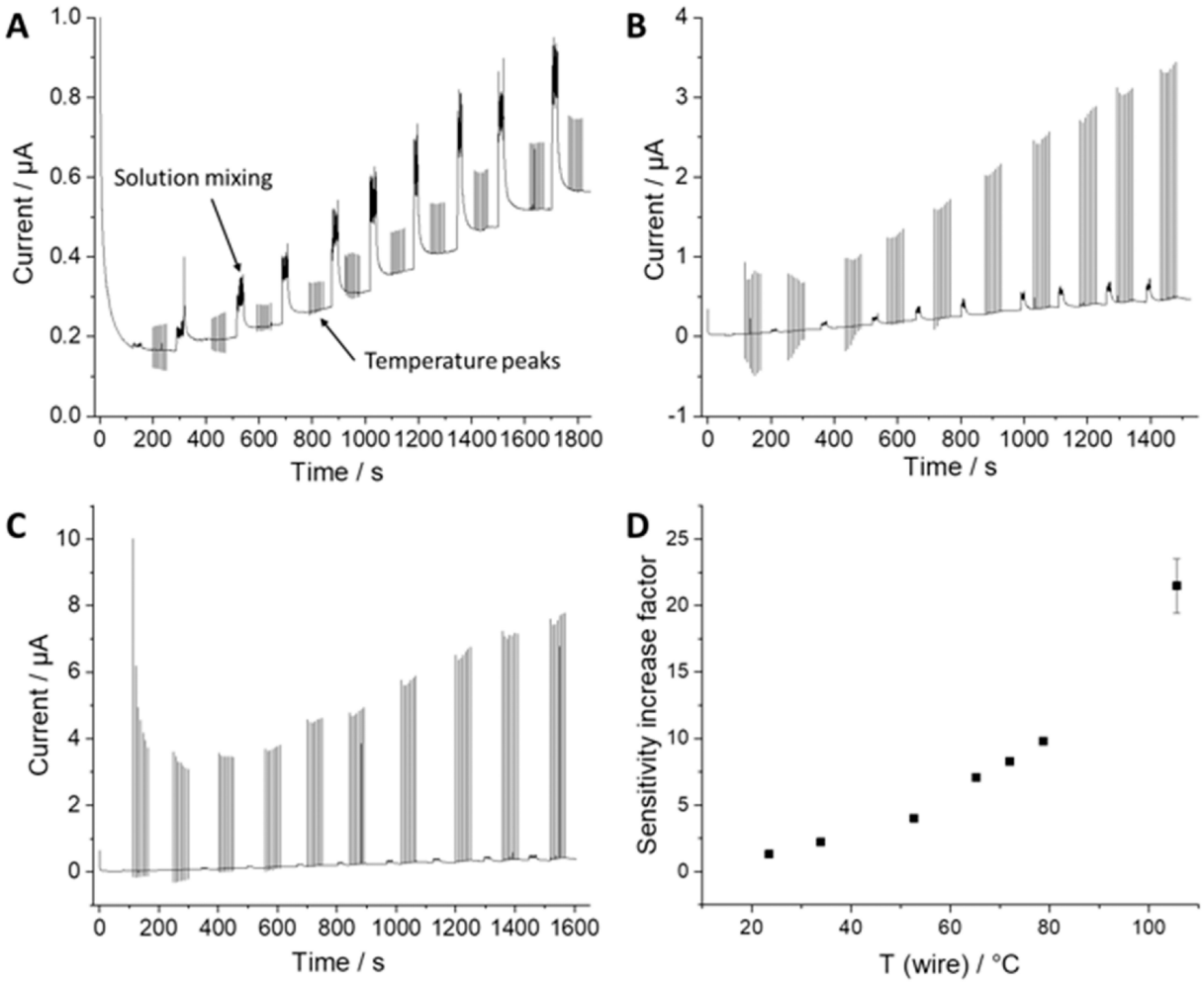

Fig. 3. A–C. Chronoamperometric measurements with a GOx-coated substrate. The wire of the substrate was coated entirely by application of 5 µL of GOx solution. Glucose concentration was increased stepwise (0.1 mM steps) from 0.1 to 1.0 mM. When a steady-state current was measured, 7 heat pulses of 100 ms duration and 8 s pulse pause were applied to the substrate. This was conducted for every concentration step with pulses leading to wire temperatures of 24 (A), 65 (B) and 106 °C (C). D: Sensitivity increase factor determined for different wire temperatures. The factor is given by the ratio of peak currents vs. currents measured 0.5 s prior to heat application.

±2. The reason for this error can be seen in Figure S6 in the SI, where the signal increase factors are given for every glucose concentration and temperature. It is shown that for 106 °C, the sensitivity decreased with higher glucose concentrations. This could be attributed to thermal inactivation of the enzyme due to the high intensity and increasing number of heat pulses applied to the substrate, as together with the stepwise concentration increase, more pulses were applied to the substrate in total. This observation is also in line with the decrease of currents during the first pulses in Figure 3C. In summary, temperature pulses of up to 79 °C were applicable to the substrate without noticeably damaging the GOx coating. In a similar study, T.-F. Tseng et al. [17] achieved a 24-fold increase of sensitivity when applying 68 °C for 30 s. This might be attributed to different immobilization protocols for GOx. In ref. [17], GOx was embedded in a Nafion membrane. However, our results show that higher temperatures can be applied when the pulse duration is decreased.

The localized activity changes of GOx spots upon heat application was studied by means of SECM. Moreover, the impact of long-term heating for 30 s was compared to the application of a single 100 ms heat pulse. First, a spot on the wire was located by using both feedback mode imaging in FcMeOH and G/C mode imaging in glucose solution. The feedback mode allowed distinguishing the wire from the surrounding substrate due to conductivity differences. The G/C mode showed the location of the GOx since it produced H_2O_2 as electroactive species that was collected at the probe. Figure 4A and B show the feedback and G/C mode SECM images of the wire and the enzyme spot, respectively. In the feedback image, the wire is visible as a band of elevated current with a width of 100 µm as its high conductivity results in an elevated response. The G/C image shows an increased current at

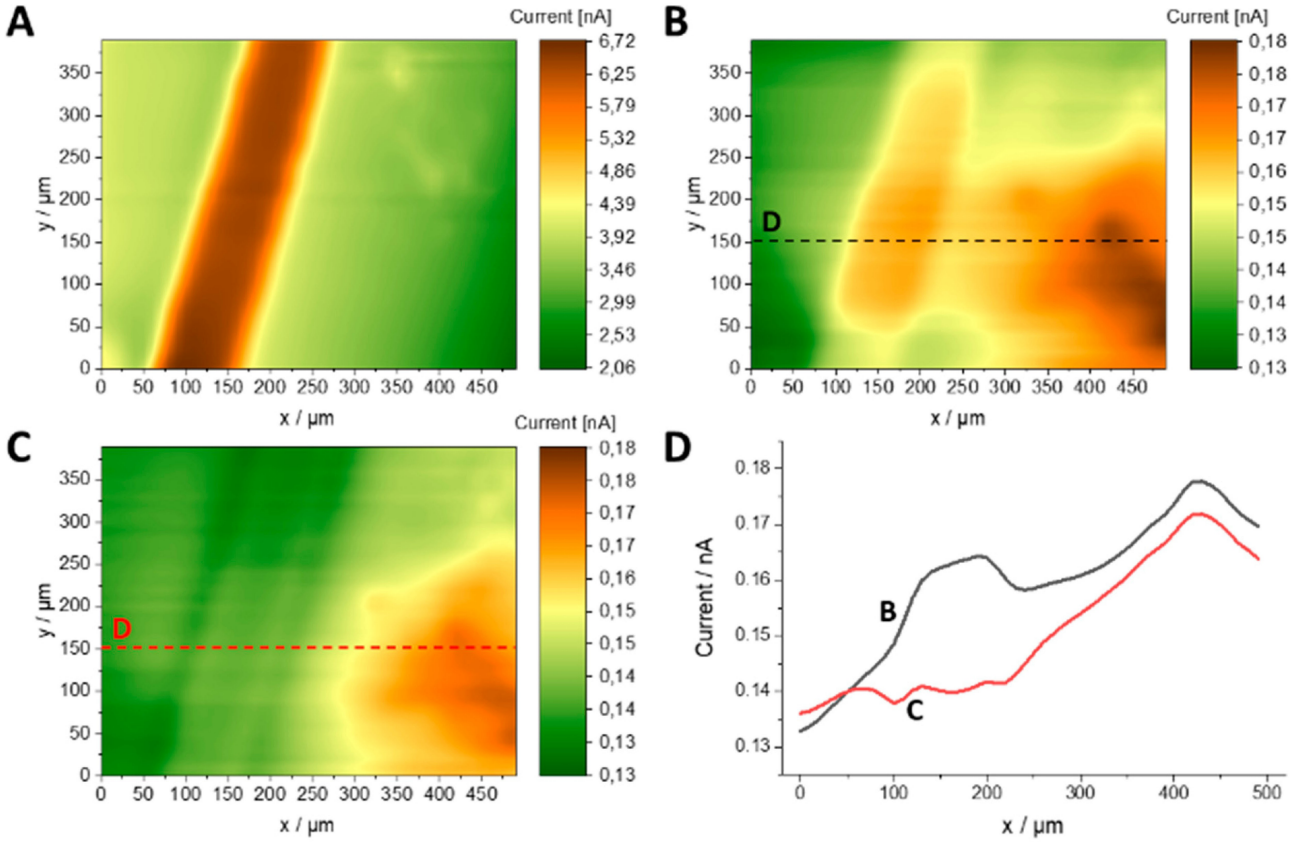

Fig. 4. SECM images at the Pt wire of the substrate where a spot of GOx was applied. A: Feedback mode image in 1.5 mM FcMeOH. B/C: G/C images recorded in 10 mM glucose prior to heating (B) and after applying 42 °C for 30 s at the substrate (C). Lines within both images have been extracted and are compared in D. An area covering 400 µm x500 µm was investigated. Pixel size: 10 µm. Wire-to-tip distance: 14 µm. E (Probe) = 0.5 V (A), 0.8 V (B–D).

the location of the wire. The current to the right of the wire is higher than to its left, indicating that most of the enzyme is located on the right side. GOx is also immobilized directly on the wire, shown by higher currents measured in its middle part. At the edge of the wire, lower currents were measured, probably due to an inhomogeneous enzyme distribution within the drop-coated spot. Overall, the shape and size of the enzyme spot correlates well with the microscopic image in Figure 1D. In Figure 4C, another G/C image is depicted, which was recorded after applying a wire temperature of 42 °C for a period of 30 s to visualize the consequences of long-term heat application. Compared to the previous G/C image, the orange area corresponding to high currents considerably decreased in size. Most notably, no increased currents were measured directly at the wire, indicating that most of the GOx activity was lost close to the wire due to a temperature gradient within the entire substrate, with the highest temperature at the wire surface.

Figure 4D shows extracted lines from both G/C images for better visualization of the consequences of continuous heating. Line B shows a current plateau corresponding to the high GOx activity directly on the wire. Further to the right, another current peak occurred which is in the center of the enzyme spot. After heat application, the current decreased along most of the extracted line as shown in line C. The most significant drop in the collector current signal occurred directly at the wire, which was most likely caused by a temperature gradient, as the highest temperatures were present directly at the wire. In addition, the thermal convection that resulted from heating possibly had a stirring effect inside the measurement cell. As a result, the H_2O_2, previously accumulated close to the GOx where it was generated, was spread throughout the solution. Afterwards, less H_2O_2 was produced by the GOx due to the damage from heating, and lower currents were measured.

For comparison, the impact of pulsed heating on GOx activity was studied as well. Figure 5 shows SECM images of another enzyme spot on a newly prepared substrate. This time however, a single 100 ms heat pulse with a maximum temperature of 106 °C was applied to the substrate. Figure 5A and B show the feedback and G/C images obtained at a GOx spot on the Pt wire. Similar to Figure 4B, elevated currents recorded above and to the right of the wire indicate the presence of a GOx spot. After application of the heat pulse, overall higher currents were measured as shown in Figure 5C. Therefore, the

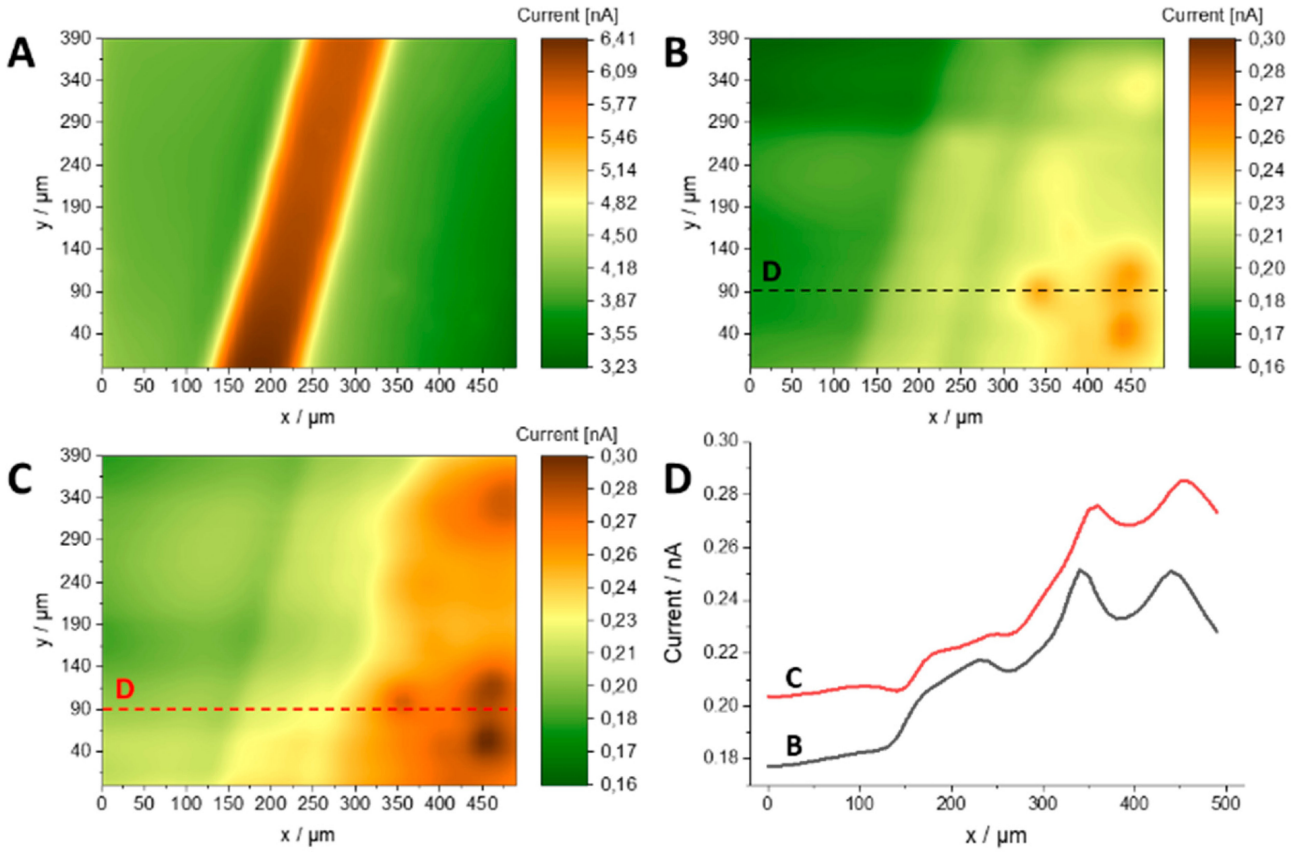

Fig. 5. SECM images at the Pt wire of the substrate where a spot of GOx was applied. A: Feedback mode image in 1.5 mM FcMeOH. B/C: G/C images recorded in 10 mM glucose prior to heating (B) and after applying a heat pulse of 100 ms duration with a peak temperature of 106 °C to the substrate (C). Lines within both images have been extracted and are compared in D. An area covering 400 µm x500 µm was investigated. Pixel size: 10 µm. Wire-to-tip distance: 14 µm. E (Probe) = 0.5 V (A), 0.8 V (B–D).

entire GOx spot was still active in terms of producing H_2O_2 to a certain degree. The extracted lines from both G/C images given in Figure 5D confirm that observation. However, the comparison of the lines shows that the current increase was lower above the wire in contrast to the remaining substrate. This indicates that the enzyme in close contact with the wire showed reduced activity, but to a less severe extent compared to the case of continuous heating. We assume that this is a result of different temperature gradients for the respective cases. In the works of Gründler [24], it was simulated that 20 ms pulses with a heating current of 725 mA led to a temperature gradient of a thickness of more than 100 µm. Under the present experimental conditions, the area around the wire that seemed affected by the heat pulse (600 mA, 100 ms) was of similar size. Therefore, we expect the temperature gradient to be of comparable size in our case.

4 Conclusion

We investigated the sensitivity towards glucose and the extent of thermal deactivation of GOx under the influence of temperature pulses. Pulses of 106 °C led to an up to 22-fold increase of sensitivity towards glucose, however decreasing with the number of repeated pulses applied indicating effects of thermal deactivation. With a pulse temperature of 79 °C, signals were enhanced by a factor of 10 without a decrease of GOx activity. Furthermore, the spatially resolved thermal deactivation was investigated using SECM. G/C mode images of the enzyme immobilized on the wire showed that 106 °C applied for 100 ms led to a lower degree of thermal deactivation compared to 42 °C applied for 30 s. Overall, these results show that the sensitivity of glucose biosensors can be further increased by the application of very short temperature pulses and decreasing the pulse duration allows to apply higher temperatures as the extent of enzyme degradation decreases.

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Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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