Electrochimica Acta 330 (2020) 134998

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Electron transfer from FAD-dependent glucose dehydrogenase to single-sheet graphene electrodes

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ARTICLE INFO

Article history:
Received 12 June 2019
Received in revised form
25 September 2019
Accepted 2 October 2019
Available online 6 November 2019

Keywords:
Graphene
Monolayer
Direct electron transfer (DET)
Glucose dehydrogenase (GDH)
Nitrosoaniline

ABSTRACT

Continuous glucose monitoring (CGM) is an emerging technology that can provide a more complete picture of the diabetes patient's glucose levels. Amperometric blood glucose tests typically require redox mediators to facilitate charge transfer from the enzyme to the electrode, that are not ideal in CGM settings because of their potential toxicity or long-term stability issues. Direct electron transfer (DET) would eliminate this need and has therefore attracted substantial interest. However, most DET-based glucose biosensor studies so far have used glucose oxidase (GOx) leading to controversial results because the oxygen dependency may be misinterpreted as DET. Here, we overcome this challenge by using an oxygen-insensitive glucose dehydrogenase (GDH). To enable direct electron transfer, the enzyme was immobilized on the surface of high-quality single-layer graphene electrodes via short pyrene linkers (< 1 nm). The biosensor strongly responded to glucose even without a redox mediator, implying direct electron transfer. Control measurements on different surfaces further confirm that the response is enzyme-specific. The activity of immobilized enzymes was confirmed by glucose measurements with a conventional ferrocenemethanol mediator as well as relatively unexplored redox mediator - nitrosoaniline. The influence of a most potent interferent in blood, ascorbic acid, was assessed. This is the first demonstration of application of single-layer graphene electrode to obtain DET from an oxygen insensitive enzyme (GDH), highlighting the potential of such devices for applications in CGM.

1. Introduction

Diabetes is a group of metabolic disorders characterized by increased blood sugar or glucose levels, which, if left untreated, can cause severe long-term complications. On the other hand, hypoglycemia - low blood sugar concentration, caused *e.g.* by medications used to treat diabetes, can result in *e.g.* loss of consciousness [1]. Therefore, measuring glucose levels is important to ensure proper disease management for those diagnosed with diabetes. Glucose biosensors have been under development for more than 60 years since the first report by Clark and Lyons [2]. In this so-called "first generation" glucose biosensor, the enzyme glucose oxidase (GOx) catalyzed the oxidation of glucose aided by oxygen as an electron acceptor. In the case of first generation GOx biosensors, the glucose concentration is measured indirectly *via* monitoring the decrease in local oxygen concentration or increase in hydrogen peroxide concentration (a product of the enzymatic reaction). The main principle, *i.e.* dependence on and correlation with oxygen concentration, was at the same time a drawback, as the oxygen concentrations in a sample may vary leading to false results. The oxygen molar concentration in *e.g.* venous blood does not exceed 0.2 mM [4] and varies significantly with different conditions. To overcome this issue, oxygen was substituted with an artificial electron acceptor, a mediator, that can be sensed at the electrode. Biosensors relying on artificial mediators to shuttle electrons between the enzyme and the electrode are generally referred to as "second generation" biosensors [3]. An example of a "classical" redox mediator for enzymatic determination of glucose is ferrocene, first demonstrated in 1984 [4]. With a smart choice of mediator and the immobilization matrix for the enzyme, researchers were able to lower the effect of interferences present in complex samples (*e.g.* blood). The trend that has emerged over the

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https://doi.org/10.1016/j.electacta.2019.134998
0013-4686/

last 30 years is to eliminate the mediator and "wire" the enzymes' active center to the electrode to transfer the electrons directly ("third generation") without the need for any mediator. This phenomenon, called "direct electron transfer" (DET) became a "Mount Everest" for (bio)electrochemists because it would enable studies of direct enzyme-substrate interactions, not limited by the use of additional mediator, and reduce the complexity of the system. DET could be highly advantageous for Continuous Glucose Monitoring (CGM), since so far CGM is based on oxygen as means of cofactor regeneration due to high requirements imposed on implantable devices (e.g. non-toxicity requirements exclude the use of mediators). Recently, Bobrowski et al. discussed the long-term implantable biosensors including CGM systems [5]. Commercially available CGM devices are based on oxygen as means of cofactor regeneration (1st generation e.g. Eversense [6]) or an enzyme entrapped in a redox polymer matrix (2nd generation e.g. FreeStyle Libre [7]). Although there has been much research on DET in glucose biosensors, most researchers have used oxidases that are oxygen sensitive. Unfortunately, it is difficult to distinguish the proposed DET from the oxygen-mediated pathway, rendering some of the DET claims rather contentious in the light of recent critical reports [8,9]. While many articles claim DET from GOx, the sole glucose determination method is based on the decrease of oxygen reduction current. Some authors do underline the fact that this is "first generation" biosensor (e.g. Ref. [10]), some do not (e.g. Ref. [11]). To circumvent this issue, a possible solution would be to use an oxygen insensitive enzyme such as glucose dehydrogenase, which has been lately shown to facilitate DET [12]. A recent review discusses direct electron transfer of FAD-dependent dehydrogenase complexes, with a special interest in glucose dehydrogenase [13]. The applications of glucose dehydrogenase (GDH) have been very limited so far partly due to the enzyme's substrate specificity issues [14]. However, progress in enzyme engineering has led to highly specific GDH that, according to the manufacturer's specification sheet for the GDH used here [15], has sufficiently high specificity against other sugars such as maltose, galactose and xylose.

Due to its intriguing properties, graphene has attracted particular interest since its discovery by Geim and Novoselov [16,17]. Graphene has been shown to be highly sensitive to its environment and considered for biosensing applications, because of its atomically thin structure and extraordinary high charge carrier mobilities. Also, monolayer graphene has been shown to be suitable and advantageous for use in electrochemistry due to fast electron transfer kinetics [18,19]. Quite recently, Kwak et al. proved that it is possible to construct a flexible graphene-based enzymatic glucose sensor on PET foil [14]. On top of that, graphene offers the ability to easily pattern electrodes in different shapes, including 3D structures and flexible substrates, that may be advantageous in designing new biosensor configurations, *e.g.* for CGM devices.

So far, the term "graphene" in the context of electrochemical glucose biosensors claiming DET has been used to describe different materials including *e.g.* reduced graphene oxide [20], graphene oxide [21], nanosheets [22], nitrogen doped graphene [23], quantum dots [24], mesocellular graphene foam [25] or simply flakes [26] with a single exception of graphene grown by chemical vapor deposition (CVD) and transferred onto a glassy carbon electrode [27]. The advantage of this material over frequently used (reduced) graphene oxide is its high quality, resulting in higher conductivity, more control over surface modification *via* non-covalent pyrene chemistry, and reduced probability of competing reactions at defect sites. Previous works have claimed that graphene may facilitate DET by: 1) "plugging in" a carbon nanomaterial to access the active site, 2) partially unfolding the protein (GOx) enabling the direct electron transfer to the electrode or simply 3) having a high surface area with excellent conductivity [28].

In this work, we used CVD grown single-layer graphene directly as the working electrode (WE). The aim of this work is to investigate the electron transfer from GDH to single-layer graphene electrode. Due to the small linker size (<1 nm), DET from the enzyme to the graphene electrode may be feasible. Indeed, by immobilizing GDH via short pyrene linkers ($<$ 1 nm), we measured a significant current increase with increasing glucose concentration even without a redox mediator, suggesting that DET may occur in this system. To the best of our knowledge, direct electron transfer from GDH to graphene has not been observed yet. Additional control experiments with different surfaces were performed to further support these observations and to corroborate that the response is enzyme-specific. Furthermore, the effect of a common electrochemical interference present in blood, ascorbic acid (AA), was carefully assessed and a reduction of the DET efficiency was observed in the presence of AA. Finally, we show that by adding a relatively unexplored electron mediator, a nitrosoaniline derivative (NA), the influence of ascorbic acid can be minimized.

2. Experimental

2.1. Single-layer graphene electrodes

Glass substrates ($25 \times 25 \times 1.1$ mm, Borofloat 33, Schott, Germany) were thoroughly sonicated for 15 min in acetone, isopropanol (Carl Roth, Germany) and deionized water, subsequently. After drying with N_2 stream, they were put into O_2 plasma cleaner for 10 min. Next, a metal shadow mask was used to evaporate a pattern of electrodes (10 nm thick titanium and 100 nm gold). poly(methyl methacrylate) (PMMA)After hydrophilizing the surface with another 5-min plasma treatment, Trivial Transfer Graphene pieces from ACS Material, LLC (USA) were transferred onto the glass substrates. This single-layer graphene was grown by chemical vapor deposition (CVD) on copper foil and was received with a supporting layer of poly(methyl methacrylate) (PMMA) on top of graphene and a layer of water-soluble polymer underneath. After dissolving the water-soluble polymer and transferring graphene onto patterned glass slides, the PMMA-coated graphene was left to air dry and then baked on a hot plate for 20 min at 80 °C. To remove the PMMA, the samples were put into Soxhlet apparatus overnight, which enabled cycled rinsing with freshly distilled acetone. Next, the samples were rinsed with isopropanol and baked for 20 min at 80 °C to evaporate leftover solvents.

2.2. Surface functionalization

An ethanol solution of 1 mM 1-pyrenebutyric succinimide acid (PBA-NHS, Sigma-Aldrich) was introduced into the microfluidic channel for 1 h. After that, the channel was briefly washed with ethanol and deionized water and PBS buffer (150 mM NaCl + 50 mM Na_2HPO_4, pH 7.4, Carl Roth). After that, 1 mg/mL of FAD-dependent GDH (GDH1, origin *Aspergillus oryzae* [30], BBI Solutions, UK) in PBS (pH 7.4) was introduced for 1 h.

2.3. Measurement setup

A microfluidic polydimethylsiloxane (PDMS, Dow Chemicals) channel (250 μm height, 750 μm width) with Teflon tubing was used to provide the solutions to the electrochemical setup. A CVD-grown single layer graphene was used as a working electrode, Ag/AgCl as reference electrode (DRIREF-2, World Precision Instruments) and a planar on-chip gold electrode was used as a counter electrode (Fig. 1 A, for additional view on the microfluidic electrochemical cell, see Fig. S10). The geometric surface area of

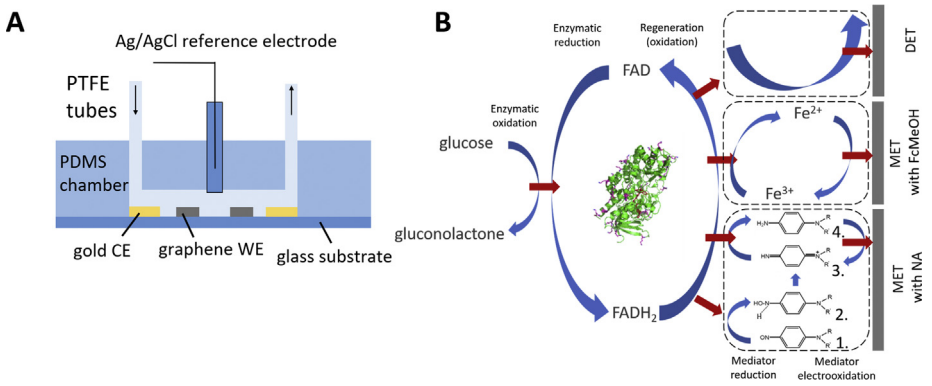

Fig. 1. A Microfluidic 3-electrode electrochemistry setup on glass substrate with single sheet graphene as WE, gold planar electrodes as CE and bulk Ag/AgCl as RE. A PDMS microfluidic channel was placed on top. **B** Reaction scheme of different glucose biosensing pathways using glucose dehydrogenase described in this work. Path 1 is a direct electron transfer (DET); Path 2 is mediated electron transfer (MET) with ferrocenemethanol used as a mediator, Path 3 is mediated electron transfer (MET) with nitrosoaniline derivative used as a mediator. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exposed graphene was estimated to be 2.44 mm^2 and the currents were recalculated to current density using this value. The electrochemically active surface area was estimated to be $\sim$ 1.72 $\pm$ 0.1 mm^2 (see SI, Fig. S6). This difference is mainly due to passivation of graphene by PDMS for operation in liquid. Cyclic voltammetry experiments were typically performed from 0.0 to 0.5 V with 10 mV/s scan rate and the readout for calibration curves was made at 0.45 V. All the measurements were recorded using an Ivium-*n*-Stat multichannel potentiostat (Ivium Technologies) in PBS pH 7.4 at ambient temperature of 21 $\pm$ 2 °C.

3. Results and discussion

3.1. Setup and reaction mechanism

In Fig. 1 B, a schematic representation of redox reactions associated with electrochemical glucose biosensing for 3 different cases investigated in this work are presented. Glucose oxidation catalyzed by glucose dehydrogenase involves two major redox steps: 1) glucose oxidation coupled with reduction of flavin adenine dinucleotide (FAD) to its hydroquinone form – $FADH_2$ [31] and 2) active site regeneration by the final electron acceptor. In this work, the regeneration was performed: 1) directly on the electrode (direct electron transfer, DET), 2) with a conventional redox mediator (mediated electron transfer, MET) – ferrocenemethanol (FcMeOH), or 3) with an unexplored and underappreciated redox mediator (MET) - *N,N*-bis(hydroxyethyl)-3-methoxy-4-nitrosoaniline (NA).

When direct electron transfer is considered, the enzyme's cofactor is regenerated directly by transferring an electron to the electrode when a suitable potential is applied (Fig. 1 B). The analytical signal in the case of ferrocenemethanol mediator is the oxidation of Fe^{2+} to Fe^{3+} provided by the imbalance of Fe^{2+} and Fe^{3+} form caused by glucose enzymatic oxidation. In the case of the nitrosoaniline derivative (specifically *N,N*-bis(hydroxyethyl)-3-methoxy-4-nitrosoaniline), the mediator must be first pre-activated by the enzymatic oxidation of glucose in order to be sensed electrochemically. In other words, the first glucose oxidation cycle activates the mediator that can be then used for further "cycling". In presence of glucose a nitrosoaniline derivative (Fig. 1 B - 1.) is reduced to hydroxylamine in contact with the reduced form of the enzyme's cofactor (2.). It dehydrates homogenously to chinodiimine (3.), which then can undergo reduction with another simultaneous oxidation of $FADH_2$ to FAD present in the enzyme. The product of chinodiimine reduction – phenyldiamine (4.) can be electrooxidized on the electrode giving a glucose dependent amperometric signal.

3.2. Surface modification and Raman characterization

To construct a glucose specific biosensor, the graphene WE was modified with a FAD-dependent glucose dehydrogenase. First, the bare graphene was functionalized non-covalently with a well-known pyrene derivative by means of π-π stacking [32] forming a monolayer on the graphene surface (Fig. 2A and B). Here, 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBA-NHS) was chosen as a linker molecule (Fig. 2 B). The activated linker was later used to couple the NH_2 residues from surface lysines of glucose dehydrogenase (Fig. 2C). This resulted in a non-oriented covalent immobilization of the enzyme on the linker-modified electrode. Raman spectroscopy is a valuable technique to characterize a variety of parameters (doping, defects, strain, number of layers) of single layer graphene [33,34]. Raman characterization of untreated (Fig. 2 D, red), pyrenebutyric acid (PBA)-functionalized (Fig. 2 E, blue) and GDH-PBA-functionalized (Fig. 2 F, green) graphene films on glass substrates was performed. This spectroscopic technique was used to probe the quality of the graphene film and confirm the molecular functionalization in conjunction with the presented electrochemical measurements. The untreated graphene Raman spectrum (Fig. 2 D) depicts the prominent G mode peak of graphene at 1589 cm^{-1}. The low D-peak ($\approx$1350 cm^{-1}) intensity of the graphene film serves as a quality indication of the film. After the PBA functionalization of graphene, the appearance of Raman peaks associated (Fig. 2 E, blue spectrum) with a self-assembled layer of PBA on graphene [35] was observed. Following the GDH functionalization of PBA-treated graphene films, an increase in the peak intensity of Raman peaks around $\approx$1520 cm^{-1} was observed (Fig. 2 F). The latter peak of a lower intensity is also present in the PBA-treated sample.

An increasing peak intensity ($\approx$1520 cm^{-1}) with increasing GDH concentration is shown in Fig. S1. Furthermore, we also observe a consistent up-shift by one wavenumber in G peak position in each step of functionalization with the PBA and GDH functionalization. This shift in the G peak position can be correlated with the change in the charge carrier density of the graphene film (0.5 $cm^{-1} \approx 2.4 \times 10^{11}$ cm^{-2}) [36]. After the functionalization, we estimate a charge carrier density change of $\approx 5 \times 10^{11}$ cm^{-2}. Since the direction of the peak shift is towards higher wavenumber, our functionalization process has a hole doping effect on the graphene film (see Figs. S1 and S2 for further Raman characterization).

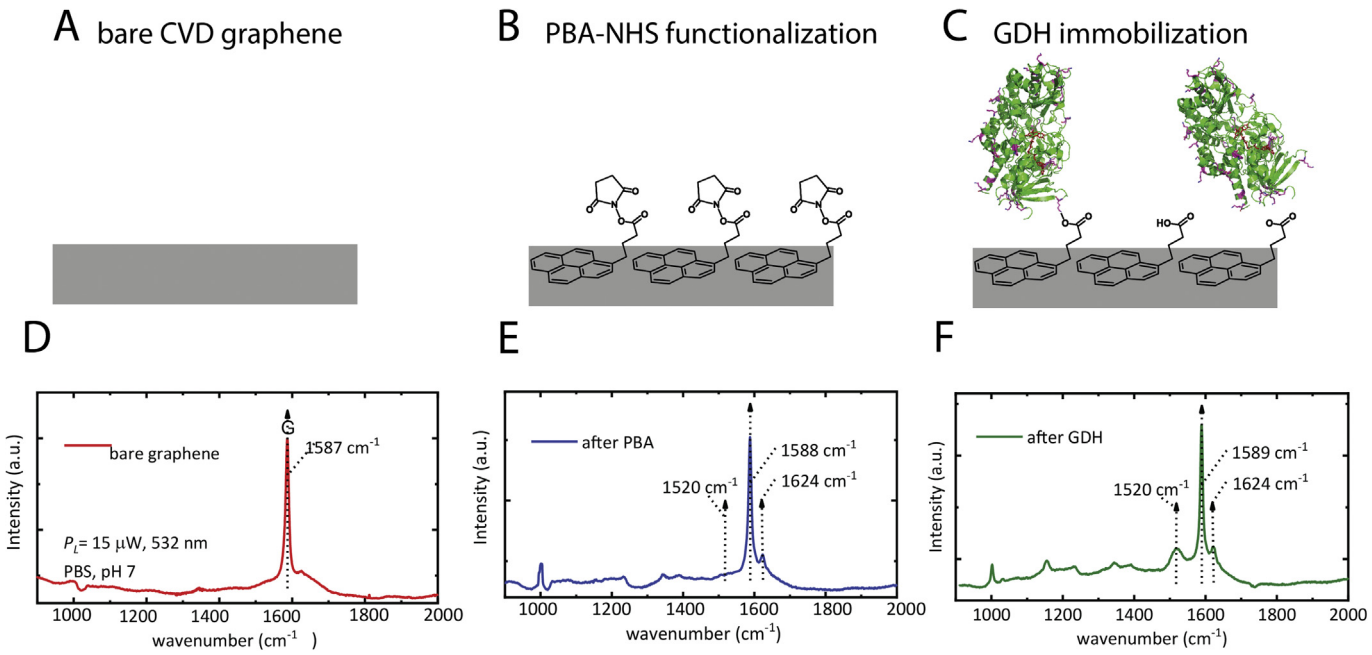

Fig. 2. Surface functionalization and enzyme immobilization scheme. **A** bare graphene electrode, **B** after incubation in 1 mM 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBA-NHS monolayer) **C** after incubation in glucose dehydrogenase (GDH). Raman spectra of bare (**D**, red), PBA-NHS functionalized (**E**, blue) and GDH functionalized (**F**, green) graphene film. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. *Direct electron transfer*

Once the surface modification and characterization was performed, we proceeded to electrochemical measurements. While performing cyclic voltammetry measurements using enzyme-coated graphene working electrodes without any redox mediator, we observed a pronounced glucose dependent signal as seen from Fig. 3 A. To make sure that this oxidation current increase was caused by the enzymatic glucose oxidation, we also performed a control experiment with bare graphene (Fig. 3 B). No current increase is observed. Instead, the apparent oxidation current decreases slightly with increasing glucose concentration, which may be due to impurities on the graphene surface such as quinones [37].

The calibration curves for bare and GDH modified graphene electrode are plotted in Fig. 3C. Since there is no current increase without enzyme, we conclude that the glucose dependent current increase observed with the enzyme-coated graphene must be due to the enzymatic activity, even though no redox mediator is present in solution. This behavior may be explained by direct electron transfer (DET) from the active center to the electrode. To test the reproducibility, these measurements were repeated with another device, showing similar behavior (Fig. S3).

To further investigate the DET pathway and to make sure that the pyrene linkers do not contribute to the glucose oxidation, we perfomed glucose calibration measurements with pyrene-coated graphene (Fig. S4). There is no glucose dependent increase in

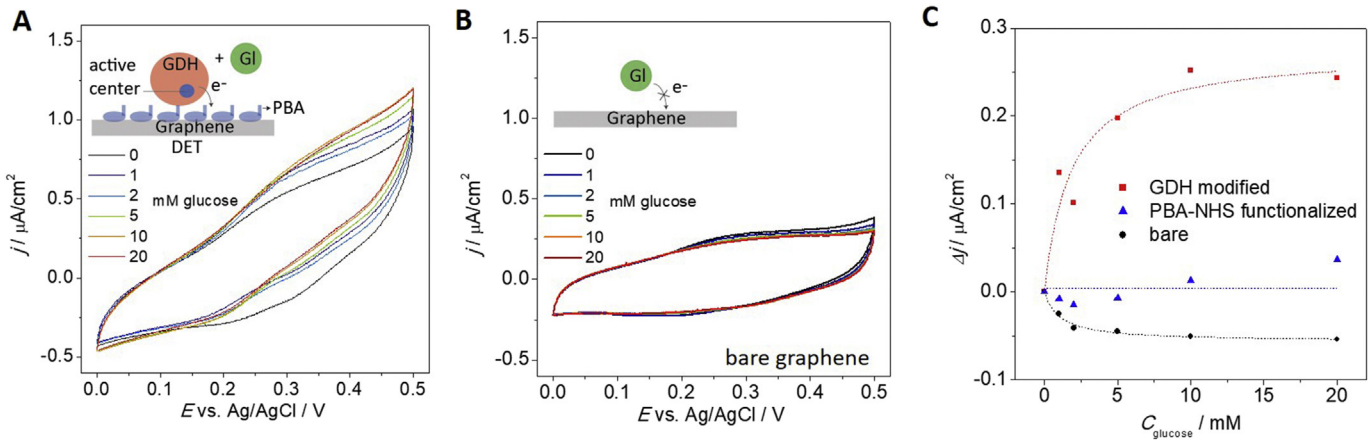

Fig. 3. Direct electron transfer from the cofactor of GDH on graphene/PBA-NHS/GDH electrode. **A** Typical cyclic voltammetry curves of non-mediated enzymatic glucose oxidation (10 mV/s, PBS pH 7). Inset: schematic representation of DET on graphene coated with PBA-NHS and GDH. **B** Typical cyclic voltammetry curves of bare graphene electrode in presence of different concentrations of glucose (10 mV/s, PBS pH 7). Inset: schematic representation the bare graphene in contact with glucose solution. No DET occurred in this case. **C** Calibration curves of glucose biosensing using direct electron transfer on graphene/PBA-NHS/GDH electrode. A glucose concentration current increase is clearly seen for GDH modified electrode, while for both bare graphene and PBA-NHS modified electrode (for cyclic voltammograms see Fig. S4) the currents do not change.

current with PBA-NHS coated graphene electrode. The calibration curve is plotted in Fig. 3C. Overall, two different control experiments were performed - 1) with bare graphene (Fig. 3 B) and 2) with PBA-NHS coated graphene (Fig. S4). These control experiment confirms that only when the enzyme GDH is present on the surface (Fig. 3 A), the monitored current is increasing. This provides additional evidence that the electron transfer is enzyme-specific and can occur without addition of a redox mediator.

During a glucose measurement in blood, electrochemical interferences may occur and lead to incorrect glucose values. Therefore, the influence of interferences must be carefully considered when developing a glucose sensor. One of the most severe sources of interference in electrochemical blood glucose measurement is ascorbic acid (AA) [38] with 0.08 mM upper limit of reference values in plasma [39]. Without AA, a clear glucose dependent signal was observed from the GDH to the electrode (Fig. 3 A). We performed measurements of both bare (Fig. 4 A) and PBA-NHS + GDH coated graphene electrodes (Fig. 4 B) in presence of 0.2 mM AA. There was significant ascorbic acid oxidation current, higher for the case of bare graphene (Fig. 4 A) indicating that the PBA-NHS functionalization along with further GDH immobilization partially passivates the surface of graphene. These results are promising, because they indicate that additional passivation strategies, such as addition of poly (ethylene glycol) [40,41], may be used in future work to further suppress the interaction with the interferents.

3.4. Mediated electron transfer using ferrocenemethanol as a redox mediator

To test if the immobilization protocol preserved the activity of the enzyme (and thus provide an indirect proof for DET), the conventional ferrocenemethanol mediator was added. Ferrocene and its derivatives have been already employed with glucose oxidase and with glucose dehydrogenase (NAD-GDH [42], PQQ-GDH [43], FAD-GDH [30]). We exposed the aforementioned biosensor (graphene/PBA-NHS/FAD-GDH) to solutions with increasing glucose concentration and performed cyclic voltammetry measurements. The voltammogram has a shape typical of reversible one-electron redox reactions. The peak-to-peak separation is $\Delta E_p = 121$ mV and the formal redox potential is $E^\circ = 0.232$ V vs. Ag/AgCl (3 M KCl), which is in agreement with previous reports $E^\circ = 0.239$ V vs. Ag/AgCl (3 M KCl) [30]. For a redox process involving one electron, the theoretical value of the peak-to-peak separation should be 59 mV, which indicates that the resistance of the microfluidic electrochemical cell is relatively high. As one can see in Fig. 5 A, there is a ferrocenemethanol oxidation peak increase with increasing glucose concentrations, while the reduction peak of ferrocenemethanol is barely affected by the increasing concentration of glucose in the solution.

As one can see in Fig. 5 B, a significant oxidation current increase was observed for buffer containing ascorbic acid and ferrocene. In comparison with the buffer without AA, the reduction peak of ferrocenemethanol was suppressed, and the oxidation peak current is 3 times higher (without *vs.* with AA). On top of that, the curves' peak currents and potentials are not as predictable as the ones for the curves recorded without AA. To make a calibration curve, the absolute current density increase (Δj) was considered at 0.45 V (in diffusion-controlled regime of the electrooxidation reaction), which is defined here as follows: $\Delta j = j_{glucose} - j_{blank}$, where j_{glucose} is the oxidation current density in buffer with the given concentration of glucose and j_{blank} without any glucose. The calibration curve is plotted in Fig. 5C.

The calibration curve for the case without AA presents a typical Michaelis-Menten behavior, while for the case with AA the calibration curve is not predictable in a straightforward manner. Additionally, the difference between the first concentration tested and the "blank" is much higher indicating some additional chemical reactions in the system.

Overall, using ferrocenemethanol as an electron mediator confirmed that the enzyme was successfully immobilized and retained its activity on the surface. However, upon exposure to a model interferent, ascorbic acid, the performance of the biosensor to distinguish different concentration of glucose dropped significantly. The recorded voltammograms with ascorbic acid showed that there is some interaction between ferrocene derivative and ascorbic acid itself leading to unpredictable and high oxidation currents and lack of a reduction peak. The catalytic behavior of ferrocene derivatives towards ascorbic acid oxidation is well-known [44] and shown in Fig. S5.

Finally, using the Randles-Sevcik equation and taking the voltammograms recorded with bare graphene electrode with varying scan rate (Fig. S6) one can calculate the electroactive surface area to be $\sim 1.72 \pm 0.1$ mm^2. The same electrode was also used to confirm that the glucose itself is not electrooxidized on the surface of graphene.

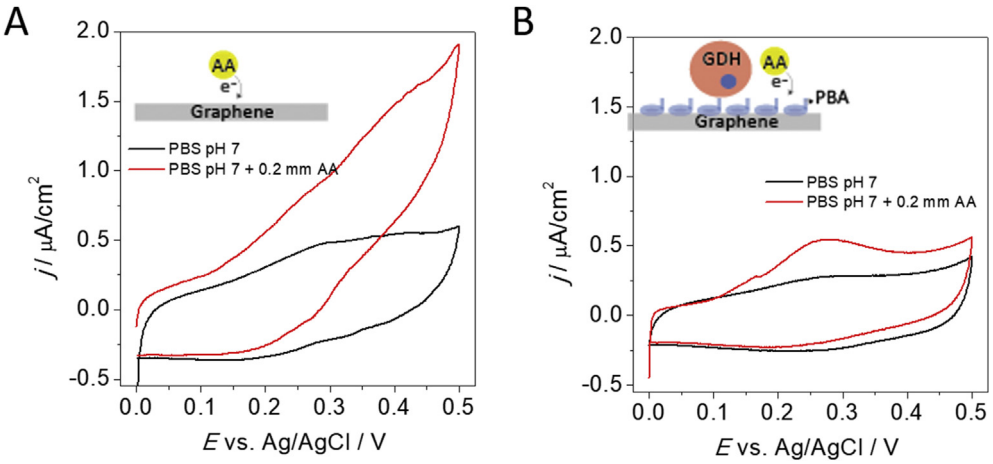

Fig. 4. Ascorbic acid oxidation on graphene electrodes: **A** bare graphene, and **B** GDH modified graphene electrode. The ascorbic acid oxidation currents are higher for the case of bare graphene (**A**) than for the GDH modified graphene indicating that GDH immobilization as well as PBA-NHS functionalization of graphene passivates the surface of graphene.

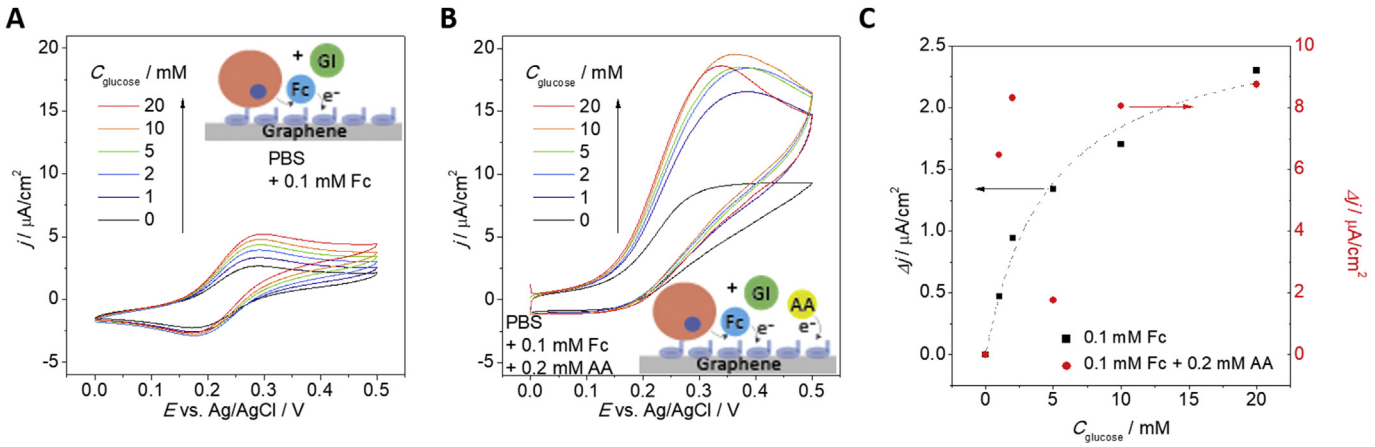

Fig. 5. Ferrocenemethanol as a conventional redox mediator in glucose biosensors. **A** Typical cyclic voltammetry curves of FcMeOH mediated glucose oxidation – an increase in glucose concentration increases the FcMeOH electrooxidation signal (scan rate: 10 mV/s, solution: 0.1 mM FcMeOH in PBS pH 7) **B** Typical cyclic voltammetry curves of FcMeOH mediated glucose oxidation in presence of 0.2 mM ascorbic acid. **C** Calibration curves of glucose biosensing with FcMeOH with (red circles) and without (black squares) 0.2 mM ascorbic acid in solution. As one can see, a substantial relative current increase with increasing glucose concentration is seen for the case with ascorbic acid. The "background" current, coming from FcMeOH oxidation-reduction is highly interfered by ascorbic acid – the reduction peaks are supressed and the oxidation peaks are not easily predictible. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Mediated electron transfer using nitrosoaniline as a redox mediator

Next, we employed a nitrosoaniline derivative as a mediator, which is used commercially by Roche Diagnostics in the glucose strips.

The electrochemistry of nitrosoaniline derivative on graphene electrode is presented in Fig. S9. The formal redox potentials for nitrosoaniline, specifically phenyldiamine-chinodiimine redox pair, are $E^{\circ} = 0.128$ V vs. Ag/AgCl (3 M KCl) and the peak-to-peak separation is $\Delta E_p = 170$ mV.

We recorded several cyclic voltammograms (CV) in pure PBS and in PBS with 10 mM nitrosoaniline (Fig. 6 A). Both CV measurements overlap proving that without the substrate (glucose) there is no redox signal increase. Afterwards, we examined the influence of ascorbic acid on nitrosoaniline oxidation (Fig. 6 B). With AA only in solution, we see an ascorbic acid peak at ~ 0.25 V vs. Ag/AgCl. When NA is added, we observe that the oxidation of ascorbic acid and nitrosoaniline add up without catalytic effects. Also, the ascorbic acid oxidation peak disappears in favor of the overall shape determined by nitrosoaniline oxidation cyclic voltammogram. This suggests that although no catalytic effect is seen, ascorbic acid reacts with nitrosoaniline activating it, which is seen as current increase coming from electrooxidation of phenyldiamine.

Next, we performed several cyclic voltammetry experiments with increasing glucose concentration using graphene/PBA–NHS–GDH electrode in presence of nitrosoaniline (Fig. 7 A). There are no clearly developed oxidation peaks, yet an overall oxidation current increase with increasing glucose concentrations is seen. For the case with AA (Fig. 7 B), one can see a very similar trend of current increase offset by ~ 3 μA/cm^2. The calibration curve of the current increase *vs.* glucose concentration is plotted in Fig. 7C and the results obtained for the case with and without AA match each other. The same behavior was found in a repetition of this experiment with another device (Fig. S7).

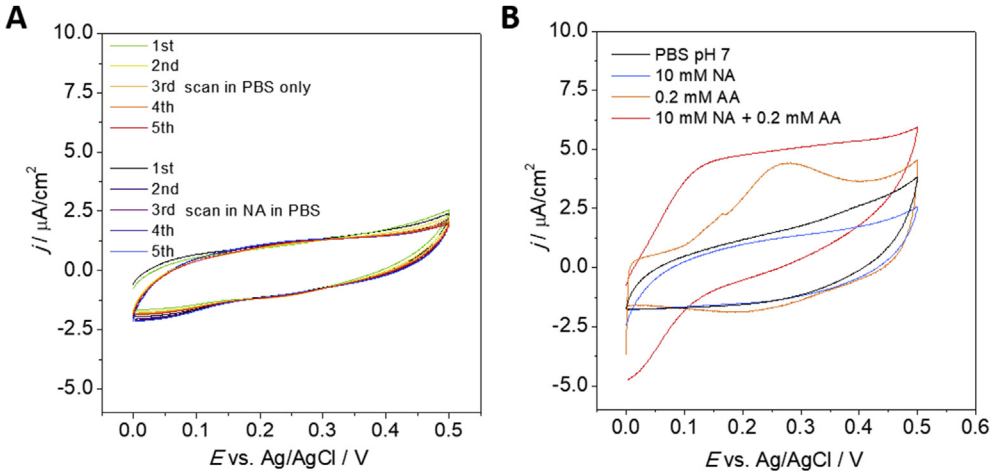

Fig. 6. Nitrosoaniline electrochemistry on graphene. A Consecutive (1–5) cyclic voltammograms of G/PBA–NHS–GDH electrode in PBS pH 7 and in 10 mM NA (10 mV/s) B Cyclic voltammograms of G/PBA–NHS–GDH in pure PBS pH 7 (black), 10 mM NA (blue), 0.2 mM AA (orange) and 10 mM NA + 0.2 mM AA (red) in PBS pH 7. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

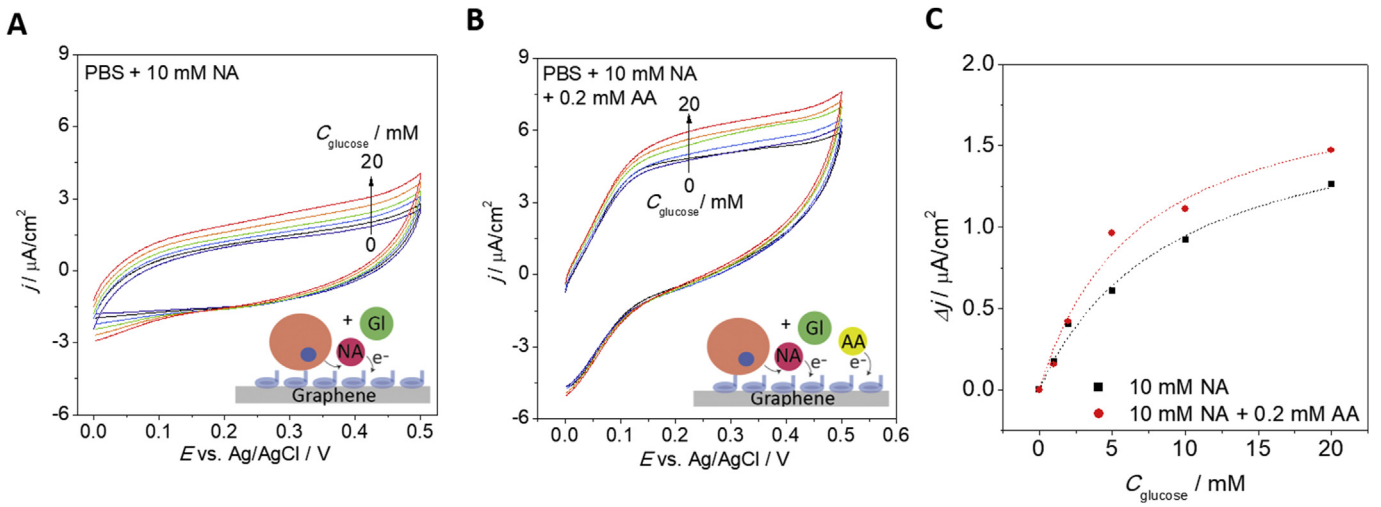

Fig. 7. Nitrosoaniline as a redox mediator in glucose biosensors. **A** Typical cyclic voltammetry curves of NA mediated glucose oxidation (10 mV/s, 10 mM NA in PBS pH 7) **B** Typical cyclic voltammetry curves of NA mediated glucose oxidation in presence of 0.2 mM ascorbic acid. 10 mV/s, 10 mM NA in PBS pH 7 **C** Calibration curves of glucose biosensing with NA with and without 0.2 mM ascorbic acid in solution.

3.6. Discussion

Here, an enzymatic electrode consisting of a single-layer graphene functionalized with glucose dehydrogenase was used for glucose determination. We evaluated different pathways of glucose enzymatic oxidation on graphene electrodes: 1) based on direct electron transfer (DET) and 2) based on mediated electron transfer (MET) with ferrocenemethanol and 3) based on mediated electron transfer (MET) using a relatively unexplored and underappreciated nitrosoaniline (NA) derivative (*N,N*-bis(hydroxyethyl)-3-methoxy-4-nitrosoaniline). Compared to conventional mediators such as ferrocenemethanol, nitrosoaniline and its derivative, are much less affected by cross-reactivity with interferents [45–47]. On the other hand, ferrocenemethanol is readily available to act as an electron mediator and produces more electrochemical signal, whereas nitrosoaniline must be pre-activated first, which is done enzymatically and thus limited by enzyme turnover. The limiting step of the NA reaction mechanism seems to be the enzymatic activation of nitrosoaniline to chinodiimine. The electrooxidation reaction rate is higher than the enzymatic activation, which is confirmed by the lack of oxidation peaks of phenyldiimine. As a result, there are no "background" redox peaks as in the case of ferrocenemethanol, which means that any recorded current increase will be associated with glucose oxidation. A more detailed discussion of the NA reaction mechanism explaining the lack of redox peaks is provided in the SI.

Regarding the DET pathway, a recent editorial in *Biosensors and Bioelectronics* discussed the inability of native glucose oxidase to undergo direct electron transfer [9]. The most explored, yet unconvincing evidence for it is based on direct electrochemistry of $FAD/FADH_2$ pair about −450 mV *vs.* Ag/AgCl. These characteristic peaks were also observed by Vogt *et al.* [48] with redox titrations showing that the formal potential for free FAD at pH 7.4 is −0.417 V *vs.* Ag/AgCl and −0.302 V *vs.* Ag/AgCl for the bound FAD (expected when considering DET). The higher value is also consistent with spectrophotometric studies of bound FAD [48]. In a recent critical article [8] the authors argue that most studies claiming direct electron transfer on nanostructured electrodes were not carried out carefully and conscientiously. It cannot be excluded that the glucose detection mechanism in a majority of the articles claiming DET on graphene may be based on O_2 depletion, rather than on DET. This would classify these reported devices as "first generation" rather than "third generation" glucose biosensors. Therefore, it was postulated [9], that there is a need to develop new enzymes, in particular dehydrogenases, since they are oxygen independent. A review article by Bollella et al. [49] further discusses the importance of using different dehydrogenases as a future prospect for development of 3rd generation (DET-based) biosensors. Unfortunately, so far, the use of glucose dehydrogenases has been limited mainly due to specificity issues, yet this has been overcome with recent advances in FAD-GDH protein engineering [31]. The commercially available GDH enzyme [15] used here is highly specific for glucose, as mentioned in the introduction.

The direct electron transfer at a distance above 1.7 nm is unmeasurably small [50]. In a native glucose oxidase, the cofactor – $FAD/FADH_2$ is buried deeper than 1.7 nm, therefore a DET is very unlikely to be measured. Nano-engineered gold nanoparticle-modified electrodes with use of FAD-GDH have been reported [51]. Interestingly, in a recent publication [52], researchers proved that DET is possible with FAD-dependent glucose dehydrogenase simply covalently bound to a self-assembled monolayer modified electrode. Yet, this phenomenon is explained by the following mechanism: the FAD-GDH can transfer the electrons by means of intramolecular charge transfer from the cofactor (FAD) via iron-sulfur cluster in the catalytic subunit (3Fe-4s) to hemes in electron transfer subunit and from these directly to the electrode (the exact mechanism is described in a different work [53]). A notable advancement was also published recently by the same group regarding a novel engineered FAD-GDH possessing heme subunit that enabled DET as well [54].

The microorganism of origin in this work is most probably *Aspergillus oryzae* (GDH1 from BBI Solutions as claimed by Milton et al. [30]). This suggests that there is no heme present (for more details see Ref. [55]). Therefore, we can suspect that the electron transfer from FAD to the electrode obeys a different mechanism, possibly direct tunneling. Recently, Muguruma et al. [56,57] described the use of single-walled carbon nanotube modified electrode used in combination with FAD-dependent glucose dehydrogenase. They observed current increase above the onset potential of 0.1 V (very similar to our case) and concluded that DET from FAD-GDH to SWCNTs can be explained by a quantum tunneling model, rather than through an intermediate heme step.

In our case, we used a single-sheet graphene electrode and pyrenebutyric acid as a monolayer for aminocoupling the enzyme. The average thickness of a saturated self-assembled monolayer formed by using PBA solution was measured to be 0.7 nm and the orientation of the PBA towards the graphene surface is concentration dependent [35]. Since in our experiments we used only 1 mM PBA (non-saturated monolayer), PBA is most probably "lying flat" on graphene, with the pyrene functional group parallel to the graphene surface, whereas in the saturated monolayer case, the PBA would bind at a higher angle close to 90° with respect to the graphene surface. Therefore, the maximum linker length is well below 0.7 nm, which is within the relevant range for DET to occur. It is clear that no electron transfer would occur if the active center was buried deep inside the enzyme. Unfortunately, the exact structure of the enzyme used here is not known. Due to the short linker size used, the tunneling is at least not excluded and our experimental results indicate that the process can occur in this system.

We also performed control measurements with different surfaces: bare graphene (Fig. 3 B) and PBA-NHS modified graphene (Fig. S4) and did not observe any oxidation current increase in response to glucose addition. This provides further evidence for non-mediated or direct electron transfer in the studied monolayer system. With respect to electrochemical interferences in the DET pathway, the glucose oxidation currents are lower than ascorbic acid oxidation currents. However, we think that these interfering contributions may be reduced if AA can be excluded from the vicinity of the electrode surface by appropriate surface modification, for example, by adding polyethylene glycol or a membrane. Results shown in Fig. 4 already indicate that appropriate surface modification can reduce the effect of electroactive interferents.

In a practical setting, a two electrode system can be used for sensing glucose in physiological solutions (with unknown interferents): 1) a GDH modified working electrode that would measure both the glucose specific current as well as the currents coming from interferences, and 2) a counter electrode modified with an inert entity (best similar to GDH) that does not catalyse reaction with glucose nor the elements of the sample, but measures the currents coming from the interferences. Both electrodes should additionally include sufficient passivation to reduce the effect of interferences. The two signals could be simply subtracted to give glucose specific signal. This shows that nitrosoaniline can be used in physiological samples which could not be realized with conventional (*e.g.* ferrocene) redox mediator that apparently catalytically interact with the interferents.

By fitting the data in Figs. 3C, 5 C and 7 C (without AA), we estimate the apparent Michaelis-Menten constant K_M^{app} to be 1.84 (no mediator), 4.74 (FcMeOH), and 9.11 (NA), respectively. Interestingly, Milton et al. [30] achieved similar K_M^{app}=3 mM with the same enzyme from the same source. The value of K_M provided by the manufacturer is 68 mM (free enzyme). The apparent Michaelis-Menten constant can be further increased by application of a permeable membrane applied on the surface of the assay limiting the diffusion of the substrate to the electrode [58]. Additionally, finely timed kinetic chronoamperometric measurements, where the increase of current in time is the readout, can help overcome the Michaelis-Menten kinetics limitation.

Comparing currents obtained in Fig. 3 A (no mediator) and Fig. 5 A (with FcMeOH mediator), one can note that non-mediated pathway of the glucose biosensor operation, results in a change of about 0.23 μA/cm^2 between 0 and 20 mM glucose concentration, whereas the FcMeOH mediated pathway yields a much higher change of 2.5 μA/cm^2.

This effect can be explained by the orientation of GDH on the surface of the electrode. Here, the enzyme's immobilization is random and generally we can distinguish two different types of orientations: 1) when the active center is directed towards the electrode enabling the DET and 2) all other orientations, where DET is suppressed due to long distance. Statistically, we can assume that this preferred DET-enabling orientation is less probable and some of the enzyme molecules are not directly connected to the electrode, but can remain catalytically active when a redox mediator is provided. Therefore, more enzyme molecules contribute to the MET glucose signals that to DET. To increase the DET currents, oriented enzyme immobilization may be envisioned which would require additional enzyme engineering to introduce specific binding sites [59].

4. Conclusions

In summary, high-quality large-area CVD grown single-layer graphene was investigated as a working electrode for the enzymatic detection of glucose. The electrode was modified with a monolayer of FAD-dependent glucose dehydrogenase and different paths (direct *vs.* mediated) of electron transfer were studied. A glucose-dependent increase of oxidation current was measured even in the absence of redox mediators, indicating that direct electron transfer may occur in this monolayer system owing to the short linkers used. To corroborate these results, additional control measurements were performed with bare and with linker modified graphene and no significant glucose dependent signals were recorded. To the best of our knowledge, this is the first time that a direct electron transfer from an oxygen insensitive glucose dehydrogenase to a monolayer graphene electrode surface is reported. Previous works using graphene working electrodes employed glucose oxidase instead, which is highly sensitive to oxygen, raising concerns about the robustness of DET observations to date as oxygen dependency or impurities may be misinterpreted as DET signals. By using a conventional redox mediator, ferrocenemethanol, we also confirmed that the activity of the enzyme was preserved on the sensor surface. To reduce the interfering signals from ascorbic acid, a novel redox mediator was used – a nitrosoaniline derivative. It is only activated in presence of glucose and interference effects can be easily subtracted. This is the first time a nitrosoaniline derivative was employed as a mediator on a GDH-coated single layer graphene electrode. While the measured DET currents are smaller than those in the mediated cases, the probability of DET may be further increased by more specific enzyme immobilization strategies to provide proper orientation of the enzymes. Also, the surface may be passivated by polymers such as polyethylene glycol to exclude ascorbic acid and other electrochemical interferents from the vicinity of the electrode. If above mentioned issues can be solved, the presented monolayer-based DET device may have certain advantages, for example, in continuous glucose monitoring (CGM), where the use of diffusional mediators is prohibited due to toxicity or limited long-term stability. On the other hand, the use of nitrosoaniline is justified in "regular", *i.e.* single-use, glucose test strips, because it helps to easily eliminate the effects of electrochemical interferences.

Funding sources

The research of the team "Nanomaterial-Based Biosensors" (MSF, DV, OGS and AT) at BioMed X Innovation Center is kindly sponsored by Roche Diagnostics GmbH. MJN thanks National Science Centre Poland under grant NCN 2014/15/B/ST4/04646.

Acknowledgements

The authors kindly thank Michael Hein (Roche Diagnostics

International Ltd.), Reiner Schlipfenbacher, Jürgen Spinke, Moritz Marcinowski, Heiko Mussauer (Roche Diagnostics GmbH), Arnulf Staib and Michael Marquant (Roche Diabetes Care GmbH), as well as Nesha May Andoy and Natalie Haustein (BioMed X GmbH) for fruitful discussions and comments. The authors thank Anton Malovichko and Amy Leson (BioMed X GmbH) for assistance with the design of the measurement setup. Comments and proofreading by Jana Zaumseil (Institute for Physical Chemistry, Heidelberg University) is also highly acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.electacta.2019.134998.

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