Enzyme Modified Carbon Electrodes in the Sub-Micrometer Scale

Miniaturized Amperometric Glucose Sensors Based on Polymer/Enzyme Modified Carbon Electrodes in the Sub-Micrometer Scale

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Carbon electrodes in the sub-micrometer scale were modified with polymer/enzyme films for the fabrication of miniaturized amperometric glucose sensors. The electrodes were modified by means of electrochemically induced co-deposition of various polymers and glucose converting enzymes by (i) activation of protected crosslinkers and formation of a dense crosslinker-polymer network that is able to entrap the biocatalyst on the electrode surface and by (ii) using an electrodeposition paint that was deposited on chemically modified carbon electrodes upon a basic pH shift. The polymer matrixes were equipped with Os-complexes that act as redox shuttles for the electron transfer between the electrode surface and the entrapped redox enzyme. The polymer/enzyme modified electrodes were active toward the oxidation of glucose with a sensitivity in the pA nM⁻¹ range. A linear current response was observed for a glucose concentration of up to 15 mM depending on the polymer/enzyme combination. Owing to their geometry in the sub-micron range, the developed sensors are expected to measure glucose with minimal invasiveness down to the single cell level.

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Understanding the glucose metabolism and neuronal energetics in the brain tissue under physiological and pathological conditions has been a subject of intense investigations. Glucose availability in the mammalian brain is critical for proper neuronal function and whole-body energy metabolism. Under normal metabolic resting conditions, the main consumers of glucose in the body are the brain neurons and astrocytes.

Albeit neurodegenerative diseases are not considered to be caused by dysregulated glucose metabolism, some bioenergetic disturbances appear during pathophysiological mechanisms. The reduced cerebral glucose metabolism is one of the earliest signs of Alzheimer’s disease. Obesity and/or diabetes mellitus type II have also been associated to Alzheimer’s disease progression and cognitive impairment. Additionally, the indication for perturbed glucose metabolism in Parkinson’s disease is well documented. Evidence that dopaminergic cell death in the striatum of Parkinson’s disease cases is accompanied by increased markers for oxidative stress and neuroinflammation in postmortem samples was also shown.

Detection of these target analytes and other biologically relevant species in the brain or in living cells requires sensitive and miniature techniques. A widely used method to quantify the concentration of relevant analytes in the brain is microdialysis which captures molecules from the extracellular space. However, the presence of the microdialysis probe can lead to depletion of the measured substances and thus influence their release and metabolism. Electrochemical probes in the sub-micrometer range offer several advantages owing to their size range such as high spatial and temporal resolution, low substrate consumption, minimal invasiveness and the possibility to measure even at the single cell level. Glucose oxidase (GOx) modified nanopipettes, which use the impedance change due to a pH drop at the pipette tip to verify elevated glucose levels in single cancer cells, are one example. Functionalization of conical polymer nanochannels with GOx allows for the detection of glucose concentrations down to 1 nM by measuring ionic currents. Amperometric enzyme-based microelectrodes have been used for monitoring the brain glucose level of rats. The principle of most microelectrode-based sensors described in literature relies on indirect detection via oxidation or reduction of hydrogen peroxide produced by GOx during glucose conversion. Pan et al. coated the tip of pulled glass capillaries with platinum and wax to create ring electrodes with an outer diameter of 200–300 nm. These probes are able to measure the oxidation of hydrogen peroxide and could thus be applied for analysis of the glucose level in single cells. However, these first-generation sensors are limited by the O₂ concentration and are not suitable for measuring high analyte concentrations. Moreover, high potentials have to be applied that can induce electrochemical side reactions.

A different approach is based on a mediated electron transfer between the biocatalyst and the electrode by incorporation of the biocatalyst into a redox hydrogel that is equipped with specific redox mediators. The hydrogel’s redox potential can be adjusted by choosing appropriate Os-complex modifiers, which act as mediator. Using redox polymers with low operating potentials, interferences from species like ascorbic or uric acid can be avoided. Furthermore, wiring enzymes in a three-dimensional immobilization matrix ensures a high load of the redox protein on the electrode, which is the basis for a pronounced current response of the sensor. Typically, redox polymers are used for the modification of macroelectrodes.

Miniaturization of glucose sensing probes was achieved by etching microelectrodes, resulting in electrodes with an apparent radius of 10–500 nm. Evidently, the use of a standard drop cast process, which is widely applied for macroelectrodes, for the modification of such small electrodes is hampered because of the small dimensions of these nanometer-sized objects. Hence, the modification of such miniaturized electrodes with polymer/enzyme films is challenging and requires controlled and automated deposition processes.

Here, we report on the fabrication of glucose sensors based on carbon electrodes with diameters in the sub-micron range (denoted as CME, carbon microelectrodes) that were modified with polymer/enzyme films by means of electrochemically induced deposition processes by transposing protocols that we have developed earlier for the modification of macroelectrodes to the sub-micrometer scale. The polymer/enzyme electrodes could be used as amperometric sensors for the reagentless detection of glucose.

**Experimental**

Materials and methods.—All chemicals and materials were purchased from Alfa-Aesar, Sigma-Aldrich, Acros-Orgamics, VWR or
J.T. Baker and were of reagent or higher grade. The synthesis of the Os-complex modified poly(1-vinylimidazole-co-allylamine)-[Os(bpy)2Cl](PVI-Os) was described earlier in Ref. 26. The Os-complex precursor Os(bpy)2Cl2 (bpy = 2,2′-bipyridine) was synthesized according to Ref. 27. The preparation of the amino modified Os-complex {Os(bpy)2BimNH2}[PF6]2 was described in Ref. 28.

The polymerization and characterization of the polymer backbone poly(4-styrene sulfonate-co-glycidyl methacrylate-co-butyl acrylate) (P(SS-GMA-BA)) was described elsewhere. The nominal composition of the polymer backbone is SS = 50 mol%, GMA = 30 mol% and BA = 20 mol%. The acetylated dithiol-based crosslinker 2,2′-(ethylenedioxy)-bis-(S-acetylated) (CL1) was prepared according to Ref. 30. The crosslinker PEGDGE (poly(ethylene glycol) diglycidyl ether) was purchased from Sigma Aldrich and revealed an average Mn of 500 Da.

UV-vis absorption measurements were conducted with a Cary60 spectrophotometer from Agilent in quartz or poly(styrene) based cuvettes with an optical path length of 1 cm.

Electrochemical experiments for the characterization of the redox polymer P(SS-GMA-BA)-Os were conducted with a portable PalmSens2 v4.4 potentiostat with a standard three electrode configuration. The counter electrode was a Pt wire and the reference electrode was a Ag/AgCl/3 M KCl system. As working electrode graphite electrodes with a nominal diameter of 4 mm were used. Polymer films were prepared by a standard drop cast process from aqueous solutions.

Electrochemical measurements with sub-micron carbon electrodes were carried out at room temperature using a two-electrode system with a Ag/AgCl/3 M KCl counter/reference electrode. Unless otherwise stated, all potentials were reported with respect to this electrode. Experiments were performed using either a VA−10 potentiostat (npi) or an Axopatch 200B Patch clamp amplifier (Molecular Devices). Experiments were performed using either a VA−10 potentiostat (npi) or an Axopatch 200B Patch clamp amplifier (Molecular Devices). The experimenter wore electrostatic discharge protection gear during the etching process. Thus, for each CME a slightly different behavior was observed, which might stem from the pyrolysis procedure and/or from the polymerization and crosslinking of the polymer backbone, CMEs were immersed in a 10 mM PB (pH 7.9) electrolyte solution containing 3 mg mL−1 PVI-Os, 0.85 mg mL−1 PQQ-GDH, 0.85 mg mL−1 PQQ-CDH and the following pulse sequence was applied: n(0 V/1.5 s; −2 V/250 ms) with n = 10. Prior to polymer/enzyme deposition, the CMEs were modified with amino groups by potential cycling in 4.7 mM 1,7-diaminoheptane (1.7-DAH)/0.1 M LiClO4/EtOH(H2o) electrolytes with a scan rate of 20 mV s−1 following protocols described in Ref. 33. For purification, the DAH-modified CMEs were successively immersed in ethanol and ultrapure water (20 min each).

Results and Discussion

Fabrication of graphite electrodes in the sub-micrometer range (CME).—The use of electrodes with dimensions in the sub-micrometer level is a prerequisite for the in-vivo analysis in living cells. Recently, it was demonstrated that such nanometer-sized electrodes can be formed from laser-pulled quartz capillaries by pyrolytic decomposition of a propane/butane gas mixture. Nanocavities can enhance the stability of CME surface modifications as it was shown for Prussian Blue films that were only stable if buried inside of such a recessed cavity. Hence, the CMEs were exposed to an electrochemical etching process in 0.1 M KOH/water by cycling the potential between 1 and 2 V with a scan rate of 100 mV s−1 for 10–25 cycles (Scheme 1).

This process forms a nanocavity by removal of only carbon from the electrode tip as can be concluded from the shape of the corresponding cyclic voltammogram in [Ru(NH3)6]Cl3 solution, which exhibits a peak-shaped curve with a small peak separation: the nanocavity ensures thin layer conditions. Moreover, the formation of a cavity was further confirmed by transmission electron microscopy (TEM) (Figures 1a, 1c and 1e). Additionally, an element sensitive mapping of the electrode tip by energy dispersive X-ray spectroscopy (EDS) confirms a recessed structure. Element maps depicted in Figures 1b and 1d of as-prepared nanocavities reveal that carbon etching proceeds evenly and in form of etching fronts. The latter are found at distances of 2.35 μm (a) and 2.41 μm (d), respectively, from the orifice of the nanocavity for the two examples shown.

However, as can be seen from the TEM images (Figures 1a, 1c and 1e) of various electrodes, the glass wall of the cavity reveals randomly distributed holes/pores (bright spots in the images) of various diameters, which might stem from the pyrolysis procedure and/or from the etching process. Thus, for each CME a slightly different behavior with respect to the diffusion profiles and accessibility of the carbon electrode could be expected (vide infra) due to variations in the glass surrounding. Nevertheless, the TEM images nicely show that indeed electrodes with tips in the envisaged mm-region (average diameter of the orifice of the nanocavity: 190 ± 25 nm) were formed, which should be potentially suitable for penetration of living cells.

Redox polymers and electrochemical deposition.—Redox polymers have been widely applied for the immobilization and the concomitant electrical wiring of redox proteins to fabricate second generation amperometric biosensors. In particular, Os-complex-mediated...
fied redox polymers\textsuperscript{35} with adjusted potentials have been used for the fabrication of amperometric glucose sensors containing various glucose converting enzymes such as glucose oxidase (GOx)\textsuperscript{21,36} glucose dehydrogenase (GDH)\textsuperscript{31,37} or cellulose dehydrogenases (CDH)\textsuperscript{21} Typically, macroelectrodes were modified with polymer/enzyme films in a standard drop cast process. However, for nanometer-sized electrodes this technique is rather difficult to apply because of the complex geometry and handling of the miniaturized probe. Thus, deposition processes, either based on an electrochemically induced crosslinking of polymer backbones by the in-situ activation of protected crosslinkers\textsuperscript{23,24,30} or by the use of electrodeposition paints for which precipitation of the polymer backbone can be induced by a pH change in front of the electrode may be applied for electrode modifications.\textsuperscript{25,38} Moreover, also the direct polymerization of conducting polymer backbones equipped with suitable redox mediators can be employed for the modification of micro- and nanoelectrodes.\textsuperscript{39} Stimulated by these previous reports, we employed redox polymers capable to undergo electrochemically induced deposition for the automated modification of the nanometer-sized carbon electrodes. In a first attempt, the Os-complex modified redox polymer P(SS-GMA-BA)-Os (Figure 2) bearing residual epoxide groups (crosslinking capabilities) and an Os-complex based mediator within the polymer backbone was used as immobilization matrix for GOx. The polymer backbone was ad-hoc synthesized in a free radical polymerization reaction between the co-monomers sodium 4-styrene sulfonate (SS), glycicyl methacrylate (GMA) and butyl acrylate (BA) with AIBN (2,2′-azobis(2-methylpropionitrile)) acting as the radical initiator.\textsuperscript{39} The amino modified Os-complex [Os(bpy)\textsubscript{2}Cl\textsubscript{2}][BF\textsubscript{4}]\textsubscript{2}\textsuperscript{28} was covalently attached to the polymer backbone via a ring opening reaction between the amino group in the complex and the polymer bound epoxide groups (Scheme S1). The UV-vis spectrum of the purified polymer (Figure S1A) exhibits the spectral signature of the complex\textsuperscript{28} indicating that the attachment to the polymer backbone has no significant influence on the electronic structure of the Os-complex itself. The redox potential of the polymer determined from cyclic voltammograms measured with drop cast films was estimated to be +0.41 V vs. Ag/AgCl/3 M KCl (Figure S1B), which is only slightly more positive than the redox potential of the freely diffusing Os-complex (+0.35 V vs. Ag/AgCl/3 M KCl).\textsuperscript{28} Residual epoxide groups within the polymer provide crosslinking capabilities that can be reacted with bifunctional nucleophilic crosslinkers such as diamines or diethylenetriamines. Such crosslinkers can be equipped with pH sensitive protecting groups that allow for an electrochemically triggered crosslinking process. For the deposition of P(SS-GMA-BA)-Os we used the acetyl protected diethylenetriamine based crosslinker CL\textsubscript{1}\textsuperscript{10} (Figure 2). By applying short positive potential pulses (+2 V vs. Ag/AgCl/3 M KCl for 500 ms) water splitting within the diffusion layer in front of the electrode occurs, which induces a pH shift to lower values and thus cleaves the pH sensitive acetyl groups. The activated crosslinker starts then to react with polymer chains and the dense 3D polymer crosslinker network precipitates on the electrode surface (Scheme 2). In the presence of an enzyme the biocatalyst is entrapped in the polymer-crosslinker network and is hence also deposited on the electrode surface. This technique was successfully used for the immobilization and wiring of GOx\textsuperscript{24,30} and also a hydrogenase.\textsuperscript{23} Moreover, the pH sensitive sulfonate groups within the backbone can be protonated during the electrochemical deposition process when positive potential pulses are applied. The protonation step decreases the solubility of the polymer chain in water and favors deposition as well. As second immobilization matrix we used the already known electrodeposition paint poly(1-vinylimidazole-co-allylamine)-[Os(bpy)\textsubscript{3}]Cl\textsubscript{2} (PVI-Os, Figure 2)\textsuperscript{26,28} which reveals a redox potential of +0.2 V vs. Ag/AgCl/3 M KCl. The polymer backbone of this polymer that was again synthesized in a free radical polymerization bears amino groups (allyl amine co-monomer) that can readily react with epoxide based crosslinkers, e.g. among others PEGDGE (poly(ethylene glycol) diglycidyl ether), \(M_a = 500\) Da, Figure 2). The imidazole units within the backbone can directly coordinate to Os-complexes containing chloro ligands. Moreover, both the amino and the imidazole units are sensitive toward pH changes. Under basic conditions (negative potential pulses) the N-bases are deprotonated and the polymer collapses due to a decreased solubility in water\textsuperscript{20} which favors precipitation on the electrode. To enhance the stability of the polymer film, the electrode surface can be modified with amino groups by electrochemical grafting of diamines in an anodic process.\textsuperscript{33} The crosslinker will then react with amino groups on the surface and with amino functions of the polymer and covalently binds the polymer layer to the carbon surface (Scheme 3, top). Further electrochemically induced deposition in the presence of crosslinker and enzyme then leads to polymer/enzyme modified electrodes (Scheme 3, bottom).
Figure 2. Proposed molecular structure of the redox polymers P(SS-GMA-BA)-Os (left) and PVI-Os (right) and the corresponding crosslinkers CL1 and PEGDGE. The redox potential of the polymer was determined from the mid-point potential of the cyclic voltammograms to be $+0.41 \text{ V vs. } \text{Ag/AgCl/3 M KCl}$ and $+0.20 \text{ V vs. } \text{Ag/AgCl/3 M KCl}$ for P(SS-GMA-BA)-Os and PVI-Os, respectively.

Polymer/enzyme modified CMEs and glucose detection.—P(SS-GMA-BA)-Os/GOx modified CMEs. For the modification of CMEs with P(SS-GMA-BA)-Os and a glucose converting enzyme, i.e. GOx, the electrodes were immersed in phosphate buffer (PB) solution of low strength (20 mM) to ensure a considerably high pH shift within the diffusion layer after applying an anodic pulse. To induce the electrochemically triggered crosslinking process the pulse sequence $n(0 \text{ V/1 s; } +2 \text{ V/500 ms})$ with $n = 10$ was applied. Figure 3a shows the cyclic voltammogram of a P(SS-GMA-BA)-Os/CL1/GOx modified CME in 20 mM PB (pH 7) in the absence (black line) and presence (red line) of glucose. Under non-turnover conditions (black line) the signals of the Os(II)/Os(III) couple of the polymer bound mediator are visible. The mid-point potential was estimated to be $+0.34 \text{ V vs. } \text{Ag/AgCl/3 M KCl}$, which is only slightly lower than the value obtained for the pristine polymer ($+0.41 \text{ V}$) in 1 M KCl/water. The peak separation of the redox-couple is 16 mV suggesting a surface confined redox reaction.

Scheme 2. Electrochemically induced crosslinking process of the redox polymer P(SS-GMA-BA)-Os. The acetyl groups of the protected bifunctional crosslinker CL1 are cleaved under acidic conditions (water splitting and thus $\text{H}^+$ generation by applying anodic potential pulses). The liberated thiols can then react with the epoxide functions in P(SS-GMA-BA)-Os in a ring opening reaction, thus forming a three-dimensional dense hydrogel network. Protonation of the sulfonate groups (green) under acidic conditions additionally decreases polymer solubility and facilitates its precipitation on the electrode surface.
couple with fast electron-transfer kinetics and only small impact of counter-ion diffusion. The electrochemical deposition process leads as expected to the formation of a polymer layer in the nanocavity of the CMEs.

After addition of glucose (30 mM, Figure 3a, red line) a catalytic wave centered at the redox potential of the Os-complex was detected. Even under turnover conditions the peaks of the Os-complex are still visible indicating that the polymer-enzyme ratio is rather large. Nevertheless, catalytically active layers could be formed via the proposed electrochemically induced deposition process. Chronoamperometric experiments at an applied potential of +0.4 V vs. Ag/AgCl/3 M KCl and increasing glucose concentrations show a Michaelis-Menten type behavior (Figure 3b) with a linear current response between 0 to 8 mM glucose. The sensitivity of the sensor was 1.61 pA mM$^{-1}$. The limit of detection (LOD), defined as three times the root-mean-square noise divided by the slope of the linear regression (Figure 3c), was 0.13 mM.

The absolute current responses measured for various sensors differ (Figure S2), most likely due to the differences in the electrode geometry and variations of the nanocavity (see Figure 1). However, all electrodes show a similar trend and an average sensitivity of (2 ± 0.7) pA mM$^{-1}$ was determined.

**PVI-Os/PQQ-GDH modified CMEs.**—The low potential Os-complex modified polymer PVI-Os was combined with reconstituted pyrroloquinoline quinone-glucose dehydrogenase (PQQ-GDH) that is in contrast to GOx insensitive to O$_2$ and thus widely used in amperometric biosensors. The lower potential of PVI-Os compared to P(SS-GMA-BA)-Os will decrease the effect of possible artefacts caused by co-oxidizable interfering compounds like ascorbic acid and uric acid (note that oxidation of these interferences at the bare CME starts at +0.15 and +0.25 V, respectively, Figure S3). For the modification of the CMEs with PVI-Os/PQQ-GDH layers, the electrode surface was first decorated with covalently bound amino groups by an electrochemical grafting process with 1,7-diaminoheptane (1,7-DAH) in 0.1 LiClO$_4$/EtOH$_{abs}$ (Figure S4). The oxidized diamines react with the carbon surface and form an amino group terminated layer on the electrode surface which can bind to a crosslinker PEGDGE. In subsequent reactions the surface linked 3D polymer/enzyme network

![3D-polymer/enzyme network](image)

**Scheme 3.** Surface modification of a CME with the diaminoalkane 1,7-DAH and covalent attachment of the redox polymer PVI-Os to the -NH$_2$/-NH decorated electrode surface via the epoxy based crosslinker PEGDGE by crosslinking the amine modified surface and the polymer chain (note that for reasons of clarity only the first reaction step to the secondary amine is depicted). By applying cathodic potential pulses OH$^-$ is generated which induces a pH shift to higher values and thus deprotonates the polymer backbone which leads to a collapse and precipitation of the polymer on the electrode surface. Moreover, OH$^-$ acts as a catalyst for the crosslinking reaction between the amines and the epoxide within the backbone and PEGDGE, respectively.

For the modification of the CMEs with PVI-Os/PQQ-GDH layers, electrochemically induced deposition PVI-Os, PQQ-GDH and PEGDGE were mixed in 10 mM PB (pH 7.9) and a pulse sequence $n(0 V/1.5 s; -2 V/250 ms)$ with $n=10$ was applied. The formation of OH$^-$ does not only deprotonate the imidazole and amino groups at the polymer backbone and thus induce polymer precipitation, but also facilitates the reaction between the -NH$_2$ groups and the epoxide crosslinker since the base acts as catalyst for the ring opening reaction.

Figure 4a depicts the cyclic voltammograms of a PVI-Os/PEGDGE/PQQ-GDH modified electrode in 100 mM PB (pH 7.1 mM CaCl$_2$). The voltammogram in the absence of glucose (black line) shows the chemically reversible signals of the Os(II)/Os(III) interconversion with a mid-point potential of +0.16 V vs. Ag/AgCl/3 M KCl which is again only slightly shifted to less positive potentials compared to the pristine polymer film. Addition of glucose (30 mM) leads to a pronounced catalytic wave with the expected sigmoidal shape and steady state currents of around 120 pA. Also, the second strategy based on electrodeposition paints ensures the formation of active polymer/enzyme layers inside the CME-nanocavity.

Chronoamperometric experiments at an applied potential of +0.35 V vs. Ag/AgCl/3 M KCl show a linear current response for glucose concentrations between 0 to 15 mM with a full saturation of the catalytic current is related to the intrinsic activity of the corresponding biocatalyst (GOx and PQQ-GDH) and also to the intrinsic properties of the redox polymer matrix, i.e. redox potential, electron conductivity, hydrophilicity, hydrophobicity and so on. Nevertheless, both sensors show a pronounced catalytic response and were able to detect glucose with sensitivities in the pA mM$^{-1}$ range. It should be noted that storing of the electrode (in buffer or in the solid state) leads to a significant decrease of the catalytic response and that the polymer/enzyme electrodes do not show a significant long term stability. However, all modified electrodes were stable within the timescale of the experiment. Thus, we conclude the biosensors are rather
Conclusions

The fabrication of miniaturized amperometric glucose biosensors based on polymer/enzyme modified carbon electrodes with dimensions in the sub-micron scale was reported. The glucose converting redox enzymes GOx and PQQ-GDH could be successfully immobilized on the surface of the electrodes by different electrochemically triggered deposition processes based on (i) the pH-induced crosslinking of an Os-complex modified polymer or (ii) the pH dependent deposition of a redox active electrodeposition paint. Both glucose biosensors were active toward the oxidation of the target analyte glucose and showed a good sensitivity and limit of detection. It can be anticipated that the proposed modification strategies may pave the way towards miniaturized amperometric glucose biosensors suitable for single-use measurements, as it is often the case for such nanometer-sized electrodes.
way to novel nano-sized analytical and biochemical tools that will allow detecting the glucose concentration at the single cell level with unprecedented temporal and spatial resolution.

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