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SURFACE and INTERFACE ANALYSIS

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Surface characterization of a cross-linked cytochrome *c* film on cysteamine-modified gold electrodes

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The aim of the present paper is to characterize a cross-linked horse heart cytochrome *c* (HHC) film on cysteamine-modified gold electrodes. The HHC film was deposited using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as a coupling agent. Attenuated total reflection infrared (ATR-IR) spectroscopic analyses were performed to characterize the newly formed surface on a qualitative and conformational level. The film thickness was measured using a noncontact optical surface profiler, while quantitative data and information on the heterogeneity of the film were obtained by means of synchrotron radiation X-ray micro fluorescence (SR micro-XRF). Results indicate that, in addition to electrochemical studies, spectroscopic analysis methods are essential to gain insight in the effect of immobilization strategies on protein conformations. The latter is of relevance in the development and optimization of biosensors.

Keywords: cytochrome *c*; EDC; cross-linking; ATR-IR spectroscopy; synchrotron radiation micro X-ray fluorescence; surface profilometry; bioelectrochemistry; biosensor

Introduction

Spontaneous or electrochemical adsorption of biomaterial onto electrodes belongs to one of the most progressive research fields in bioelectrochemistry (see Refs [1–8] and references therein). The electrical contact between the biomolecule and the electrode has its importance, among other fields, in the development of biosensors. The latter are analytical devices that detect biochemical and physiological changes. One of the most widespread examples of a commercial biosensor is the blood glucose sensor, which is used by diabetic patients.

Systems that employ enzymes as bioactive interfaces represent the most extensively studied assemblies. The design of enzyme electrodes is such that the current or potential measured is proportional to the rate-limiting step in the overall reaction. Specific attention needs to be given to the kinetics of the electron transfer (fast interfacial electron transfer is a prerequisite for optimal control) and the long-term stability (denaturation of the enzymes should not occur) of these devices, both issues can be addressed by an improved immobilization of the enzyme onto the electrode surface. For more detailed information on these various aspects, the reader is referred to the references above.

The immobilization of enzymes can be done in different ways. Possible routes include the incorporation of the enzyme into films[9,10] or the cross-linking of enzymes to form a film. The cross-linking approach of proteins generally leads to a substance with properties of a hydrogel.[11]

In the present work we aim to study, optimize and therefore also control the characteristics of a chemically cross-linked film on cysteamine (CYS)-modified gold electrodes as a primary step in the formation of potential biosensors. Horse heart cytochrome *c* (HHC) was chosen as a redox protein as it has been studied extensively with respect to direct protein electrochemistry (see Refs [4,7,12] and references therein) and has often been considered as a model system for biological electron transfer[13] and for bioelectrocatalysis.[14] The modification of the gold electrode with CYS ($HSCH_2CH_2NH_2$) was performed through chemisorption in order to achieve a covalent bonding between HHC and the gold substrate: direct contact between a redox enzyme and an unmodified electrode surface usually leads to significant structural and/or functional changes in the protein. The latter implies that the bioactivity of the enzyme is greatly reduced. The only way thus to achieve the desired interfacial properties is by modifying the electrode or protein.[15] The coupling reagent used is 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). It couples the carboxyl group of the protein with the free amine of CYS, which results in the formation of covalent amide bonds. In addition inter-protein cross-linking also occurs, which leads to the formation of a three-dimensional protein film on the electrode surface.

A previous paper by the authors reported on the optimization of the immobilization procedure and the time-dependent stability of the modified electrodes under different storage conditions.[16]

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This paper, on the other hand, aims to characterize the deposited HHC film. More specifically attenuated total reflection infrared (ATR-IR) spectroscopy was used to prove the immobilization on a qualitative and conformational level. The thickness of the film was determined using a noncontact optical profiler, while synchrotron radiation X-ray micro fluorescence (SR micro-XRF) experiments were performed to obtain a quantitative idea about the actual surface coverage and the heterogeneity of the film. These parameters are important with respect to biosensor applications since, in future work speciality enzymes and/or proteins will be incorporated in the HHC film to enable monitoring certain biochemical and/or physiological changes. As a result, a profound understanding of the characteristics of the HHC film deposited on the electrode surface is essential. Finally we would like to mention that our work fits into a 'proof of principle' experiment, i.e. by characterizing the quality of the formed film we indirectly evaluate the immobilization technique. The latter can then be used as a stepping stone for immobilizing different types of enzymes in a similar manner, thereby reaching other applications (e.g. the immobilization of cytochrome *c* peroxidase for the reduction of hydrogen peroxide). The major benefit the film will have over other protein films reported in literature is its stability.

Experimental

Reagents

HHC, 2-[4-(2-hydroxyethyl)-piperazinyl]ethanesulfonic acid (HEPES), CYS, sodium hydroxide and EDC were purchased from Sigma-Aldrich. The HEPES buffer solution of 10×10^{-3} mol l^{-1} was set to pH 7.0 using a 0.15-mol l^{-1} NaOH.

Preparation of the modified electrodes

The preparation of the modified electrodes has been described in detail in Ref. [16]. In brief the gold electrode surfaces (diameter 1.6 mm, BAS Instruments Ltd., West Lafayette, USA) were first grounded and polished to standard metallurgical procedures. This was followed by incubating the electrodes in a water solution containing 5 mmol l^{-1} CYS for 24 h at room temperature, which allowed the adsorption of CYS onto the surface. The modified gold electrodes were rinsed with water to remove any physically adsorbed CYS. In the following text, these modified electrodes are denoted as CYS|Au.

To immobilize HHC onto CYS|Au, 15 µl of a HHC containing buffer solution and 5 µl of a 2.5-mol l^{-1} EDC solution were incubated onto the surface by using a Hamilton syringe and air exposed for 2 h at room temperature. The concentration of the HHC solution was 3 mmol l^{-1}. This concentration was based on previous studies,[16] which have shown that a minimum concentration of 1.5 mmol l^{-1} of HHC is required to obtain a well-defined protein film. In order to avoid this rather delicate threshold a higher concentration of 3 mmol l^{-1} was adopted in this work. The formation of the film occurred while the aqueous phase dried. Finally, the electrodes were washed with the HEPES buffer solution. This electrode is referred to in the text as HHC|EDC|CYS|Au. Important to note is that in order to obtain the typically red colored HHC film on the electrode surface, it was necessary to expose HHC and EDC to air during the cross-linking process. The latter implied the absence of a cap on top of the electrode so that the buffer solution is able to evaporate from the formed hydrogel.

Surface analyses

ATR-IR spectroscopy

ATR-IR spectroscopic analyses were performed in order to obtain qualitative proof of the immobilization of HHC onto the modified metal surface. Transmittance spectra were acquired using a Biorad FT-IR spectrometer FTS 575C equipped with a 'Golden Gate' ATR accessory. The latter was fitted with a diamond crystal. The coating covering the electrode surface was measured directly by pressing the electrode against the ATR crystal. The spectra were recorded over the range 4000–600 cm^{-1} and averaged over 16 scans.

Optical profilometry

The thickness of the HHC film was determined using a Wyko NT3300 noncontact optical profiler. The vertical scanning mode, used in this experiment, is based on a Mirau interference microscope, where a white light beam passes through a beam splitter which reflects half of the incident beam to a reference surface and transmits the other half onto the sample. The light reflected from the sample and from the reference surface then recombines at the beam splitter to form interference fringes. The system measures the degree of the fringe modulation or the fringe contrast. During a measurement, the reference arm containing the interferometric objective moves vertically to scan the surface at varying heights. A linearized piezoelectric transducer precisely controls this motion. As the system scans downward, an interference signal for each point on the surface is recorded. A series of advanced computer algorithms are used to demodulate the envelope of the fringe signal and to extract the surface information.

Scanning SR micro-XRF

Scanning SR micro-XRF experiments were performed at Beam Line L of the DORIS-III storage ring, HASYLAB (Hamburg, Germany).[17] A microbeam size of about 20 µm [full width at half maximum (FWHM)] was obtained at an excitation energy of 7.6 keV, which was used to excite Fe-$K_{\alpha,\beta}$ fluorescent-lines without exciting Au-L lines from the (spectroscopically infinitely thick) gold substrate. The iron signal allows us to quantify the amount of protein present on the surface. Iron maps of 31×31 pixels with typically 20-µm step size were recorded using a data collection time of 10 s per data point. Quantification of the iron/protein surface involved transferring the individual pixel spectra into a sum spectrum using MicroXRF2 software and converting the measured fluorescent intensities to a concentration value including corrections for background and line overlap.[18] The nonlinear least square fitting software AXIL[19] was used to determine the net-peak intensities of the Fe-K_{α} and the background below this peak. A pure thin Fe foil (thickness 125 µm, purity 99.99+ %) from Goodfellow Inc. was used as a reference material. The actual concentrations were calculated using the fundamental parameter method.[20] Additionally the level of uniformity was determined by processing the data according to the strategy of Kempenaers *et al*.[18,21] The latter involves the following steps. The Fe-K_{α} intensity is normalized to the signal of the Au-M line, after which its distribution is examined by constructing a histogram. A Gauss curve is fitted to the histogram that allows one to determine the mean, the standard deviation and the relative standard deviation. Also this procedure allows the calculation of the protein surface coverage.

Results and Discussion

As indicated earlier on, the present paper aims to characterize the immobilized HHC layer on a gold substrate both on a qualitative and quantitative level. In the following sections, the results of the two aspects will be elaborated in detail.

Qualitative proof of immobilization

Infrared spectra were recorded on the following four electrodes: HHC|EDC|CYS|Au, EDC|CYS|Au, CYS|Au and a bare gold electrode. In addition, spectra were recorded of both an unmodified gold electrode preincubated in a HHC solution (i.e. HHC|Au) and pure HHC as control samples. Unfortunately, both the CYS-functionalized CYS|Au sample and the EDC activated CYS on the EDC|CYS|Au electrode could not be measured as the thickness of the deposited layer was too low (i.e. 1.6 nm for the CYS layer).[22] Consequently, we focused on the immobilization of HHC on the CYS-modified electrode surface.

The IR spectrum of the HHC control sample (Fig. 1(a)) shows four important characteristic peaks. The amide I mode and the N–H stretching vibration are located respectively at 1641 and 3276 cm^{-1}. The former arises almost exclusively from the C═O stretching vibration of backbone peptide linkages and is known to be sensitive to small variations in hydrogen bonding patterns and molecular geometry of proteins.[23] The latter contains information of each of the peptide residues.[24] The amide II band, which generally arises from an out-of-phase combination of N–H in-plane bending and C–N stretching vibrations of peptide linkages,[23] consists of two important peak maxima (i.e. 1541and 1511 cm^{-1}). In addition, the band near 1452 cm^{-1} can be attributed to the COH in-plane bending of the COOH side groups in the acidic glutamic acid and aspartic acid residues. The band located at 1396 cm^{-1} corresponds to C–O stretching in the HHC carboxylates. In addition to demonstrating the immobilization of the protein onto the electrode surface, IR spectroscopy is also a powerful tool to gain insight into the protein conformation.[25–28] On the basis of the literature data, the band at 1541 cm^{-1} can be assigned to a strongly hydrogen bonded NH.[29] In addition to these strong hydrogen bonds, the presence of nonhydrogen bonded amides is indicated by the peak at 1511 cm^{-1} (i.e. amide II band), whereas the peak at 1641 cm^{-1} is characteristic for the presence of hydrogen bonds located in random domains.[24,29]

The IR-spectrum of the HHC|EDC|CYS|Au sample (Fig. 1(c)) shows three peaks, i.e. at 3351, 1634 and 1540 cm^{-1}. The wave number of the amide II band shifted from 1511 and 1541 cm^{-1} to 1540 cm^{-1} after HHC deposition on the electrode surface, indicating both an increased H-bond number and strength.[24,29] The latter can be explained by the change of the heme-environment caused by a changed dielectric constant and a variation of solvation properties attributed to the covalent bonding of the HHC molecules to the gold surface.[16] The shift of the amide I band from 1641 to 1634 cm^{-1} also indicates a conformational change, from random domains toward β-strands.[23,29] The obtained IR-spectrum slightly differs from the IR-spectrum of HHC recorded by Zhang *et al* (i.e. characteristic peaks at 1654, 1548, 1448 and 1404 cm^{-1}).[30] The latter can be attributed to the fact that in that work, HHC was dissolved in a mixture of acetonitrile/phosphate buffered saline (PBS) (8 : 1), while in the present work HHC was dissolved in HEPES buffer.

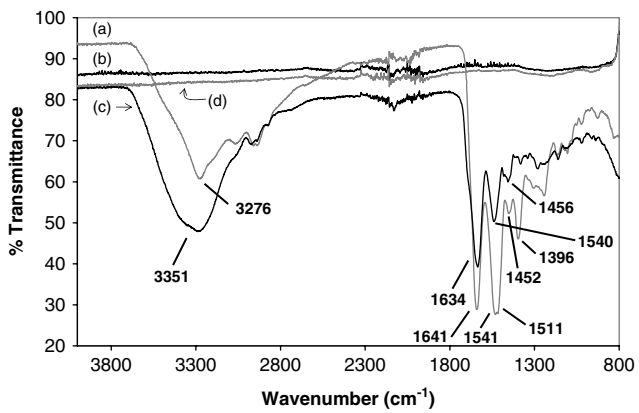

Figure 1. ATR-IR spectra of HHC powder (a), an uncoated gold electrode (b), HHC|EDC|CYS|Au (c) and HHC|Au (d).

The obtained results could be of relevance in view of potential biosensor applications, since changes in protein conformations have been reported to significantly affect their binding capacity.[31] As a result, the HHC conformation is important with respect to the incorporation of specific enzymes and/or proteins capable of detecting biochemical and/or physiological changes.

The presence of the various characteristic peaks present on the gold surface after protein immobilization clearly indicates a successful deposition of the HHC on the electrode surface. Comparison of the control sample (i.e. unmodified-gold electrode preincubated with HHC solution) (Fig. 1(d)) and the bare gold electrode (Fig. 1(b)) does not show any additional bands. The latter indicates that chemisorption of CYS, EDC activation and subsequent coupling of HHC is an excellent approach to successfully immobilize cytochrome *c* on a gold electrode. Moreover, the corresponding IR spectra of both the HHC control sample and the modified electrode (i.e. HHC|EDC|CYS|Au) are the first indication that the protein integrity is preserved upon coupling to the electrode surface. It should be noted here that the presence of characteristic peaks for various secondary structures (e.g. β-strands) does not imply that the immobilized HHC is biologically active, i.e. other techniques including electrochemistry-based approaches should be used to investigate the biological activity. However, the obtained results are the first indication that HHC can be applied as a matrix to incorporate specific enzymes and/or proteins functioning as biosensors.

Quantitative proof of immobilization

The thickness of the HHC film was determined using the 3D surface profiler. Our experiments demonstrated that it takes a few hours to obtain a stable film thickness, the time required by the buffer molecules to evaporate from the film. Figure 2 shows a 3D surface plot of the entire electrode and indicates an average thickness of 44.21 $\pm$ 0.22 µm (average of 184 $\times$ 120 data points). This relatively high value is the result of inter-protein cross-linking occurring on the electrode surface. The film thickness is affected by several parameters including the protein concentration applied, the immobilization strategy and the number of active sites (i.e. amines) on the active gold surface. In literature, several authors report on various film thicknesses for cytochrome *c* layers according to the procedure applied. For example, monolayers of cytochrome *c* adsorbed on unmodified-gold electrode surfaces have been reported to be approximately 2.2 nm.[32] Tajima *et al* obtained HHC films with a thickness of 50 nm by spincoating the 0.9 wt% aqueous solution of HHC.[33] The layer thickness is of relevance since electron transfer from the 'sensing' enzyme or

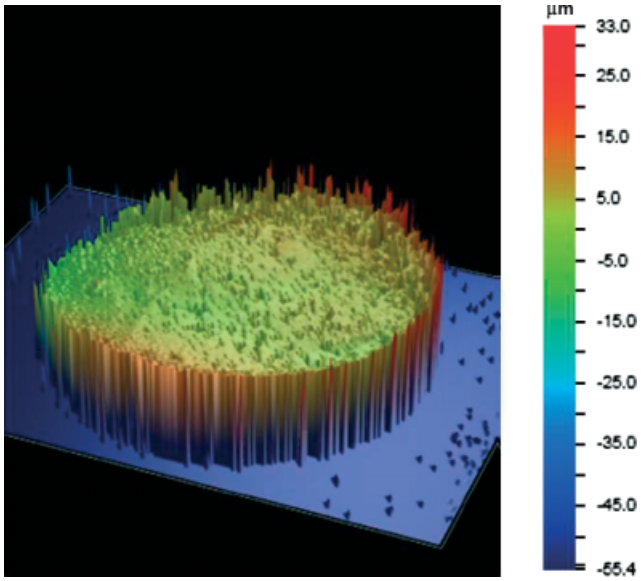

Figure 2. Thickness of the HHC film determined by a 3D surface profiler.

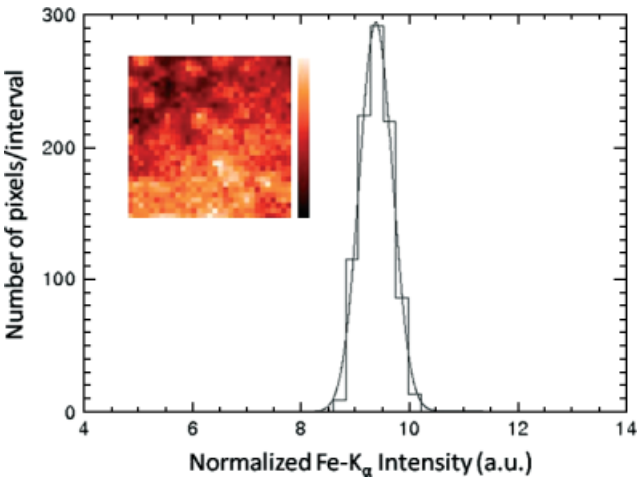

Figure 3. Elemental map of deposited Fe and the corresponding histogram of the detected Fe-K_α line obtained for HHC|EDC|CYS|Au. The XRF map consists of a 31 × 31 raster using 20-µm step size in both X and Y directions. The scale bar indicates a normalized Fe-K_α intensity between 0 and 10.4.

protein to the electrode should be retained upon incorporation in the HHC film.

The protein surface coverage was obtained by performing SR micro-XRF measurements on the HHC|EDC|CYS|Au sample. The background iron concentration was determined by performing additional analyses on the bare gold electrode, a CYS|Au electrode and an EDC|Au electrode.

Figure 3 shows the obtained elemental map and the corresponding intensity distribution of the detected Fe-K_α signal after normalization with the Au-M line for the HHC|EDC|CYS|Au sample. The histogram of the normalized iron intensity was used to investigate the homogeneity in the HHC distribution. This approach allows us to exclude possible outliers when calculating the average iron surface concentration and the associated variance. Additionally the iron concentration was also calculated on the basis of the sum spectrum of the full map. The comparison of both approaches allows us estimate the homogeneity of the iron distribution across the scanned area.

Table 1. Overview of the SR-XRF data, including the description of the modified electrode, the data derived from the sum spectrum (resp. total Fe intensity and Fe surface concentration) and the data calculated from the histograms (resp. mean of the histogram, the Fe surface concentration and the relative heterogeneity)

Samples	Surface concentration calculated using the mean of the histogram (µmol cm^{-2})	Surface concentration using the sum spectrum of iron (µmol cm^{-2})
Au	0.0012 ± 0.0001	0.0011
CYS|Au	0.0017 ± 0.0002	0.0018
EDC|Au	0.0012 ± 0.0001	0.0011
HHC|EDC|CYS|Au	0.64 ± 0.04	0.64

Table 1 summarizes the results and demonstrates in first instance that the background measurements give rise to a significant contribution of iron, namely up to 0.0018 µmol cm^{-2}. The latter was not anticipated and we assume that the contamination originates from the SR micro-XRF setup as well as from the presence of dust particles in the ambient environment. The HHC|EDC|CYS|Au sample has an average concentration value of 0.64 ± 0.04 µmol cm^{-2}. The value was obtained using both calculation methods, which indicates a very homogenous distribution of the film. Taking into account that a monolayer corresponds to 3.37 × 10^{-11} mol cm^{-2} of HHC,[10] the HHC|EDC|CYS|Au-modified electrode consists of *ca* 18 100 layers. Since HHC is a globular protein with a diameter of 2.5 µm,[34,35] it can be derived that the formed HHC film has a thickness of 45.25 µm. The latter is in very good correlation with the value obtained with the 3D surface profiler, i.e. 44.21 ± 0.22 µm.

Conclusions

This paper describes the characterization of a cross-linked HHC film on a CYS-modified gold electrode. ATR-IR spectroscopy was used to prove the actual immobilization and enabled to gain insight into the protein conformation before and after immobilization. This is of relevance in view of potential biosensor applications. ATR-IR spectroscopy is thus a valuable tool to obtain data complementary to the electrochemical results. Synchrotron radiation X-ray micro fluorescence was performed to determine the average iron surface concentration of 0.64 ± 0.04 µmol cm^{-2}. Both SR micro-XRF as well as 3D surface profilometry showed that the thickness of the cross-linked film is in the order of 45 µm. The data obtained in the present work are of relevance in the field of biosensor technology. At present, alternative methods are being evaluated to immobilize HHC on electrode surfaces including HHC encapsulation in (bio)polymers. In addition, the system developed will be applied to incorporate specific enzymes and/or proteins able to function as 'detectors' for minor changes in biological environments. The results will be the topic of the forthcoming paper.

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