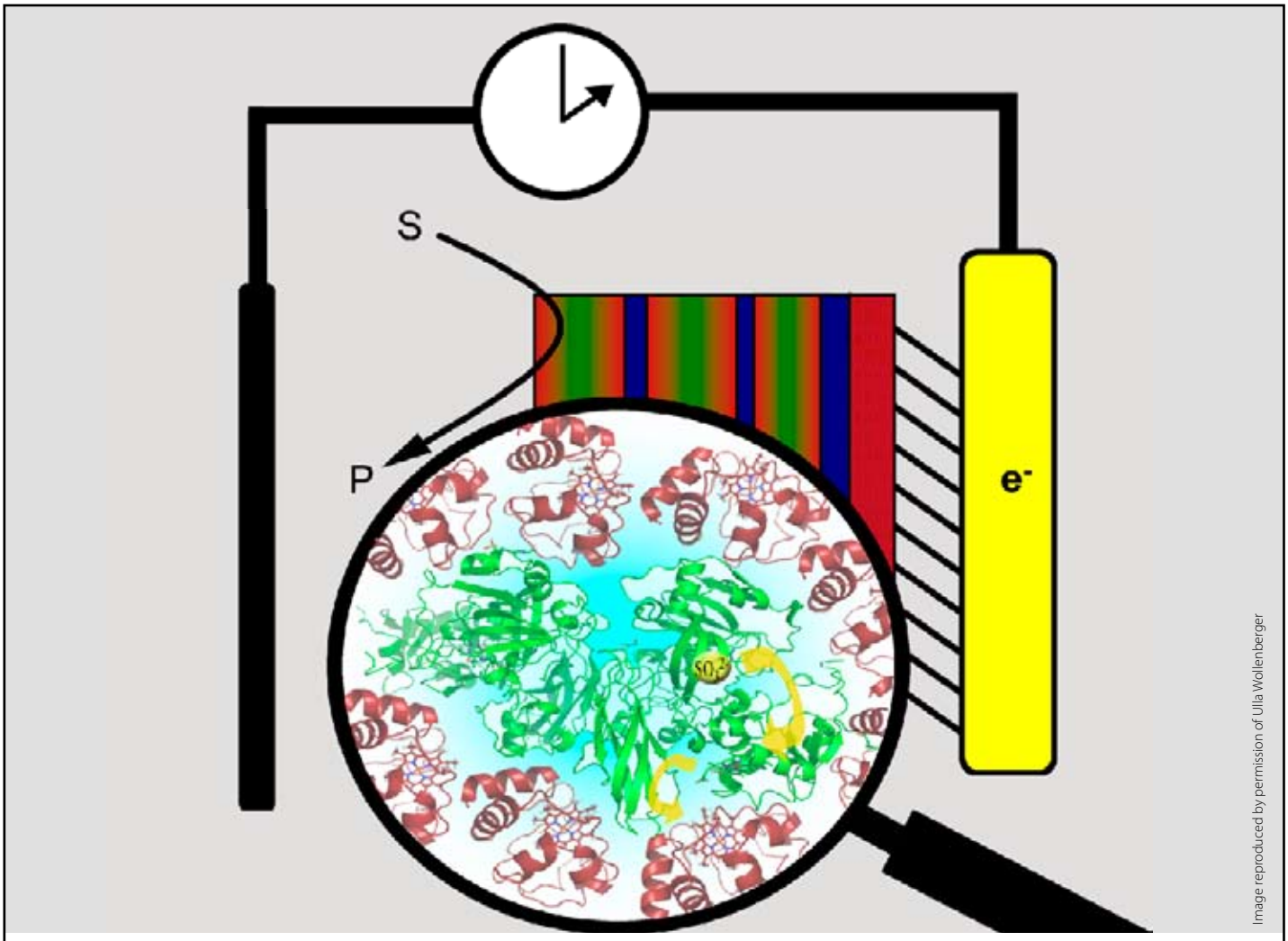

Showcasing research from the Analytical Biochemistry lab at Potsdam University.

Title: Electrocatalytically functional multilayer assembly of sulfite oxidase and cytochrome c

Electrodes with a tuned multi-layer of cytochrome c, sulfite oxidase and polyaniline sulfonate, where proteins communicate via a direct heterogeneous electron transfer and generate electro-catalytic sulfite oxidation currents.

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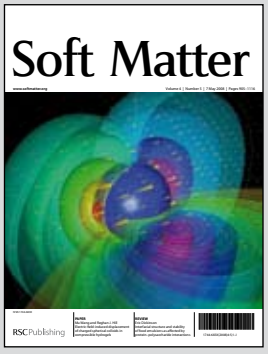

See Roberto Spricigo, Roman Dronov, K. V. Rajagopalan, Fred Lisdat, Silke Leimkühler, Frieder W. Scheller and Ulla Wollenberger, *Soft Matter*, 2008, **4**, 972.

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Electrocatalytically functional multilayer assembly of sulfite oxidase and cytochrome c†

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An electrocatalytically functional multilayer has been designed using two proteins, cytochrome c and sulfite oxidase, and a polyelectrolyte (polyaniline sulfonate). The two proteins were co-immobilized on the surface of a gold electrode in alternating layers by electrostatic interactions using the layer-by-layer technique. The formation of this fully electro-active multilayer is characterized by quartz crystal microbalance and electrochemical experiments. The electro-catalytic characterization of the device containing up to 12 layers is based on generation of an oxidation current after sulfite addition. Besides the electron-transfer mechanism, the role of the different components in the electron-transport chain is clarified. Kinetic data were extracted to characterize the multilayer function. This artificial multilayer assembly is expected to be useful in the biosensor and biofuel cell development.

Introduction

Electron-transport chains (ETC) are the main tools in the energy generation for all known forms of life. They are subsequent redox reactions that transfer electrons from an electron donor to an electron acceptor. An ETC contains a series of membrane-associated electron carriers triggering biochemical reactions that produce adenosine triphosphate (ATP), which is the energy currency of life.[1] These protein complexes are a good example of an efficient signal chain, and are the object of intensive studies in order to create an artificial system able to mimic it.[2–5]

Bio-electronic devices rely on a functional arrangement of bio-components with electronic communication. Therefore much attention has been paid in recent years to assemble electrochemically active enzymes on electrodes.[6] Many proteins, involved in inter-protein electron transfer and typically functioning in ordered structures such as bio-membranes, can be good candidates for the development of an artificial system. In fact for efficient intermolecular ET, the redox-active centres in the protein are accessible and the ET-process involves a docking process in order to reduce the ET-distance. However, the catalytic centre of many oxido-reductases reacting on small molecules is often located deep within the protein. This generally permits only a very weak interaction with the electrode. One approach to address the active site of such enzymes is based on molecular electron-transfer relays.[36]

The molybdenum cofactor-containing enzyme sulfite oxidase (SO) catalyzes the oxidation of sulfite to sulfate, the terminal reaction in the degradation pathway of the sulfur-containing amino acids cysteine and methionine. This enzyme has two cofactors present in two domains of the protein which are connected by a flexible loop: a molybdenum cofactor (Moco), where the reaction takes place, and a heme-b_5, required for the re-oxidation of the Moco.[7–10] Early approaches in biosensor construction used SO with chemical mediators,[14–16] which however can non-specifically react with other reducing agents. The heme domain is naturally re-oxidized by cytochrome c (cyt c). Thus, several publications reported on a sulfite biosensor combining the well-developed cyt c electrochemistry with the fast reaction of SO.[11–13] The behaviour of cyt c at various electrodes has been studied as well as its electron-mediating function in bioelectrocatalytic units with different enzymes.[17,18,36,37]

Cyt c is a basic protein and can therefore interact at negatively charged surfaces. This has been the basis for an efficient interfacial ET at modified gold electrodes, where it can be immobilized in a monolayer, for example, by simple electrostatic adsorption[17,18] and covalent binding,[36] or in multilayers by the usage of an anionic polyelectrolyte.[19–22] The main advantage of this assembly is the possibility to obtain a fully electro-active arrangement, where even the most distant cyt c molecules can still communicate with the electrode *via* the other protein molecules. Therefore, an increased signal can be obtained. By this technique cyt c can be attached to the surface of the electrode in a tuneable way varying the number of layers and maintaining their activity. The system described earlier[19] was achieved by just one protein, cyt c. The combination of this redox protein with an enzyme is described here. SO was selected to create a fully electro-active multilayer where electrons can flow from the substrate sulfite *via* the multi-cofactor enzyme and cyt c to the anode in sequential intramolecular, intermolecular and finally interfacial ET-steps towards the electrode. Very recently a cyt c multilayer with integrated enzyme was achieved, where bilirubin oxidase (BOD) has been co-immobilized together with cyt c by

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† Electronic supplementary information (ESI) available: Cyclic voltammogram of 0.2 mg mL^{-1} PASA in 5 mM Tris, 2 mM KCl pH 8.5 on Au-Mu/MuA. at 0.1 V s^{-1}. See DOI: 10.1039/b717694e

the layer-by-layer technique using a polyelectrolyte interlayer. The multilayer was generating an oxygen-dependent reduction current proportional to the number of layers.[22]

SO (EC 1.8.3.1) differs from BOD (EC 1.3.3.5) not only by the basic function, but also in its structural characteristics, since BOD is a monomer. SO is a homo-dimer, each sub-unit containing two flexible domains that are linked by a loop and adopt different conformations during intramolecular ET.[23] The heme b_5 has a transducer function from two-electron-transfer process to one-electron transfer and it acts between the cyt c and the Moco domain.

An artificial ETC composed of cyt c and SO formed by the layer-by-layer technique using a polyelectrolyte is described here. It represents a new system where for the first time a multi-domain enzyme and a redox protein are immobilized together and communicating in a multilayer architecture on gold electrodes by coupling of intermolecular and intramolecular ET-processes. The building strategy and the functional mechanism of the system will be elucidated.

Experimental

Materials

Horse heart cyt c was purchased from Sigma (Steinheim, Germany). 11-Mercapto-1-undecanoic acid (MUA), 11-mercapto-1-undecanol (MU) and sulfonated polyaniline (PASA) were provided by Aldrich (Taufkirchen, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium sulfite, Trishydroxymethyl aminomethane, sodium chloride, sodium phosphate, and potassium chloride were purchased from Merck (Darmstadt, Germany). Goodfellow (Bad Nauheim, Germany) supplied gold wire with a diameter of 0.5 mm. All the solutions were prepared in 18 MΩ Millipore water (Millipore, Eschborn, Germany).

Instruments

All the electrochemical measurements were performed in 1 ml cells using Ag/AgCl/1 M KCl reference (Biometra, Germany) and Pt-wire counter electrode. The working electrodes were modified gold wires. Cyclic voltammetric experiments were performed with the CH instrument (Austin, USA). A MultiLab 3900 piezoelectric instrument (J. Kitlička, Brno, Czech Republic) was used for the quartz crystal microbalance (QCM) experiments.

Buffers

Phosphate buffers were prepared from dipotassium phosphate and potassium diphosphate, with the pH adjusted with potassium hydroxide or phosphoric acid. Buffer 1: 0.5 mM potassium phosphate, pH 5, was used for the preparation of the PASA solution. Buffer 2: 0.5 mM potassium phosphate, pH 7, was used for the preparation of the cyt c-SO solution and washing. Buffer 3: 5 mM potassium phosphate, pH 7, was used for the preparation of the cyt c monolayer. Buffer 4: 5 mM potassium phosphate, pH 8, was used for the recording of the voltammograms of the multilayer electrodes. For the experiments at different pH values, a 5 mM Tris, 2 mM KCl buffer was used, and concentrated HCl and KOH were used to adjust the pH. Buffer 5: 50 mM sodium phosphate, 300 mM NaCl, pH 8 was used for SO expression and purification.

Sulfite oxidase expression and purification

His-tagged human SO was purified after expression in *E. coli* TP1000 cells containing plasmid pTG718, as described previously.[8] The cell strain containing expression plasmid was grown aerobically at 37 °C overnight in Luria-Bertani medium (LB) supplemented with 150 μg ml^{-1} ampicillin. This culture was diluted 1 : 50 into LB supplemented with ampicillin, 1 mM Na_2MoO_4 and 20 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown aerobically at 30 °C for 24 hours. After the growth, the cells were harvested by centrifugation at 8000 rpm for 5 minutes. The cell pellet was re-suspended in Buffer 5, and lysed by several passages through a French Pressure cell. After centrifugation at 13 200 rpm for 30 minutes, the supernatant was added to 1.5 mL of Ni-NTA resin (Qiagen) per litre of cell growth. The slurry was poured into a column and the extract was allowed to flow through the resin. The column was then washed with 10 resin volumes of the Buffer 5 containing 10 mM imidazole and then with the same volume of Buffer 5 containing 20 mM imidazole. The His-tagged protein was eluted with Buffer 5 containing 250 mM imidazole. Finally, the buffer of the protein was changed into 1 mM potassium phosphate buffer pH 7 by two series of dialysis and concentrated to 850 μM.

Sulfite oxidase analysis

Samples of SO were quantified spectroscopically at 413 nm using a molar extinction coefficient of 113 000 M^{-1} cm^{-1}. The activity of SO was assayed by monitoring the reduction of cyt c at 550 nm in the presence of sodium sulfite.[33]

Monolayer electrode preparation

Gold wire electrodes with a diameter of 0.5 mm were cleaned according to Katz *et al.*[35] by boiling in 2 M KOH and successive incubation overnight in concentrated H_2SO_4 and 10 minutes in concentrated HNO_3. A careful rinsing with Millipore water followed every successive step. The cleaned electrodes were incubated in a mixture of 5 mM MUA and 5 mM MU with a volume ratio of 1 : 3 for 48 h. After having been rinsed carefully with water, they were incubated in 600 μL of 20 μM cyt c in buffer 3 for 2 h and finally rinsed carefully with water and buffer 3.

Multilayer electrode preparation

Freshly prepared monolayer electrodes were successively incubated in PASA solution (0.2 mg mL^{-1} in buffer 1) and cyt c-SO solution (20 and 1 μM in buffer 2, respectively) for 10 minutes each, intermitted with three washing steps in buffer 2.

Cyclic voltammetry

Cyclic voltammograms of the electrodes were recorded in buffer 4 at scan rate of 5 mV s^{-1} between −200 and +200 mV unless noted otherwise.

QCM experiments

A clean gold-covered quartz sensor chip (10 MHz, ICM, USA) was incubated in a mixture of 5 mM MUA and 5 mM MU with a volume ratio of 1 : 3 for 48 h. Then the chip was rinsed with Millipore water and mounted into the QCM flow system. Cyt c-SO and PASA solutions and buffer were used as described for the multilayer preparation. The solution containing cyt c-SO and PASA were successively pumped through the cell for 10 minutes with 5 minutes of buffer in between, at a flow rate of 20 μL min^{-1}. The change of the frequency signal *versus* time was recorded.

Results and discussion

Formation of the multilayer

The SO-cyt c multilayer is formed on a monolayer of cyt c, obtained by protein adsorption on a mixed self-assembled alkanethiol layer on gold electrodes.[24] The overall charge of the thiol monolayer is negative due to the carboxylic groups of the self-assembled mercaptoundecanoic acid. The electrostatic interaction between the negatively charged electrode and the positively charged protein surface permits the immobilization of the cyt c without blocking its mobility, and thus provides a good communication not only with the electrode but also with the proteins of the surrounding solution.

The formation of the SO-cyt c multilayer was confirmed by quartz crystal microbalance (QCM), which correlates the increased surface mass after each alternating deposition with the frequency change. After the monolayer formation, the modified gold chip was flushed sequentially with the PASA and a solution containing one SO per 20 cyt c molecules, intermitted by buffer (0.5 mM potassium phosphate, pH 7). The addition of the SO-cyt c mixture generates a large and fast increase of the mass on the surface of the electrode, which was followed by a partial displacement of material after the PASA solution was pumped over the surface. Fig. 1 shows the cumulative net increment, which was achieved after each cycle.

The role of polyaniline sulfonate (PASA) is to act as an anionic polyelectrolyte that facilitates the immobilization of cyt c layers by electrostatic forces.[19,25] SO can be co-assembled with cyt c at an appropriate pH on the basis of their different

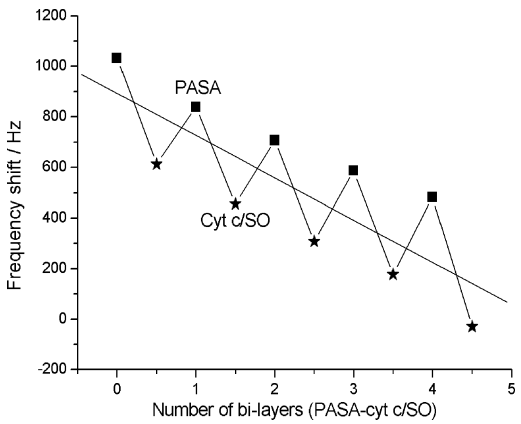

Fig. 1 Frequency shift for five successive deposition rounds of SO-cyt c (stars) and PASA (squares) on a monolayer of cyt c modified gold chip (quartz crystal 10 MHz).

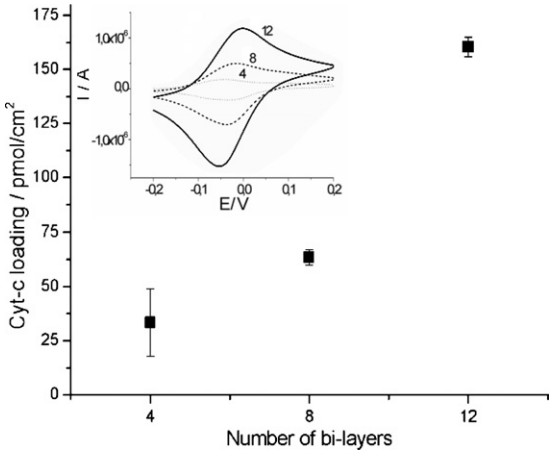

Fig. 2 Dependence of the amount of electroactive cyt c on the number of bilayers n of a Au/MU,MUA, (cyt c,SO/PASA)$_n$ multilayer electrode. Inset: CVs of multilayer electrodes at 0.1 V s^{-1} for an increasing number of bilayers. Error bars result from 3 replicated measurements.

iso-electric points, of 5.5 for SO and 10.5 for cyt c. At pH 7 it is possible to obtain a strong interaction of the two proteins with a higher SO incorporation than at pH 5. Thus, the former pH was used for the multilayer formation.

In preliminary experiments, other immobilization strategies were tested, but the best SO incorporation was achieved by using a solution containing both proteins. For example, imobilizing the two proteins in separated layers lead to a not fully electro-active multilayer (not shown).

When the multilayer, however, is prepared from a mixed solution of both proteins, cyclic voltammetry (see Fig. 2 (inset)) shows a significant increase in oxidation and reduction peak currents with growing numbers of layers, which corresponds to an increase of the amount of electro-active cyt c molecules immobilized by each deposition step. From an integration of the faradayic peak currents, the amount of cyt c per layer communicating with the electrode has been calculated to be 10.9 ± 2.5 pmol cm^{-2}.

Functional description – catalysis

The catalysis of the sulfite oxidation within the multilayer can be followed by CV at slow scan rates (inset Fig. 3). When a non-limiting concentration (20 K_m) of sulfite is added, the CVs transformed into sigmoidal anodic waves. With a further increase of the concentration of sulfite, no increase in the response was observed. The catalytic current however is increasing with the number of layers. This current behaviour clearly indicates an electron-transport chain from the substrate *via* the proteins to the electrode. Under the assumption that the catalytic reaction at the enzyme is the rate limiting step (see the ET mechanism section below) the limiting current I_{lim} can be expressed as a function of the amount of SO[11] present in the multilayer:

$$I_{lim} = k_{cat}\Gamma_{SO}nAF \quad (1)$$

In the equation n is the number of electrons exchanged, Γ_{SO} is the coverage of SO on the electrode surface, F the Faraday constant and A the surface area. The k_{cat} value for the enzyme

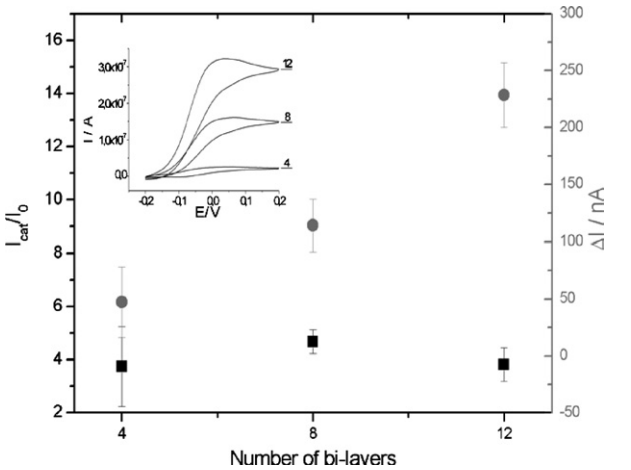

Fig. 3 Effect of the number of bilayers on the catalytic oxidation current of a Au/MU,MUA, (cyt c,SO/PASA)$_n$ multilayer electrode. The ratio of catalytic oxidation and non-catalytic current (black squares) and the absolute current generation (grey circles) are depicted in dependence on the number of bilayers. Inset: CVs of multilayer electrodes after the addition of 1 mM sulfite with 4, 8 and 12 bilayers.

Table 1 Reaction and electron-transfer rate constants in solution.

Process	Rate/M^{-1} s^{-1}
SO (k_{cat}/K_m)	$\sim 2 \times 10^{7a}$
SO-cyt c reduction	$\sim 4 \times 10^{6a}$
Cyt c–cyt c self-reduction	$\sim 1 \times 10^{3b}$

[a] Determined within this study [b] Ref. 30

reaction with the electron acceptor was determined in solution (see Table 1) and used for the calculation of the coverage of SO actively communicating with the electrode.

For instance, from the intensity of the current under substrate saturation recorded at 5 mV s^{-1} (Fig. 3), $n = 1$, and from the k_{cat}, spectroscopically evaluated in the same assay buffer just before the immobilization, it was possible to estimate the Γ_{SO} per layer to be 0.14 ± 0,02 pmol cm^{-2}. From the analysis of this value compared to the amount of immobilized cyt c per layer, the ratio between the two proteins within the layer structure was determined (~ 1/77). The difference between the SO–cyt c ratio calculated here and applied during the multilayer formation (1/77 and 1/20, respectively) could be explained by the use of the k_{cat} value determined for both proteins in solution, although in the multilayer assembly the enzyme reacts in the immobilized state.

However, the linearly growing amount of SO with increasing numbers of bilayers also indicates that the SO molecules immobilized very far away from the electrode are still contacted to the electrode by the cyt c molecules. At a fixed number of layers the catalytic current is also dependent on the substrate concentration (Fig. 4).

The situation in the multilayer largely differs from the reaction in solution, particularly with respect to the diffusion within the protein assembly. Sulfite is a rather small molecule, and can diffuse through the multilayer structure, however the diffusion across the multilayer is slower than in the solution, where a very small K_m was determined (~ 1 μM). A sulfite gradient can be

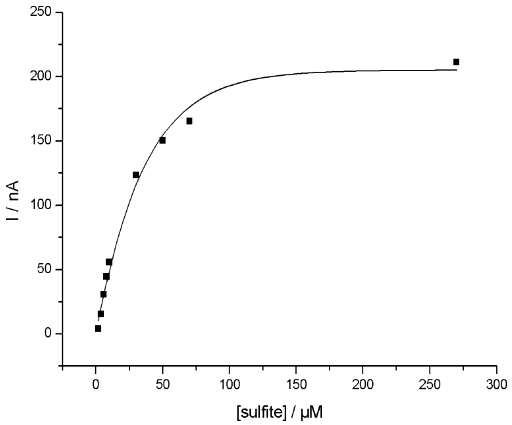

Fig. 4 Dependence of the catalytic oxidation current generated by a SO–cyt c multilayer electrode (8 bilayers) on substrate concentration (applied ratio of SO–cyt c = 1 : 20; E = +100 mV *vs.* Ag/AgCl).

formed from the bulk solution to the electrode. Therefore an increase of the apparent K_m value (K_m^{app}) for the SO-cyt c multilayer is expected. From the current measured at different sulfite concentrations (Fig. 4) and the Lineweaver–Burk graph the K_m^{app} value for an eight-biprotein-layer electrode was calculated to be ~ 56 μM. The sulfite saturation concentration of 1 mM used for the further studies was quantified by this value (≈ 20 K_m^{app}).

SO–cyt c ratio

The mechanism of the developed functional unit involves the generation of two electrons at the Moco-site by sulfite oxidation, which are subsequently transferred in two 1-e-transfer steps to the heme b_5 and then further to cyt c. The electrons are then transferred most probably *via* a fast self-exchange to the polarized anode, where finally cyt c is re-oxidized.

The SO-cyt c ratio is an important factor to verify this model and to optimize the catalytic efficiency. Keeping the cyt c concentration at 20 μM, the SO quantity was varied and three different parameters were analyzed: the amount of electroactive cyt c, the ratio between catalytic and non-catalytic current and the absolute current generation.

The amount of electroactive cyt c immobilized on the electrode is diminished in the presence of higher SO concentrations (about 70% in the presence of 4μM in comparison to 0.25 μM SO). The ratio of the current in the presence and absence of the substrate is an indicator for the efficiency of the SO-cyt c communication. It is slightly enhanced going from 0.25 to 4 μM SO but decreases with higher SO concentrations (Fig. 5). The highest catalytic current is generated when 1 μM SO has been co-adsorbed with 20 μM cyt c (Fig. 6).

In summary it can be stated that at SO concentration below 1μM, the total amount of immobilised enzyme is too low to generate a significant catalytic current, whereas at higher concentrations (≥4μM) the amount of cyt c actively communicating with the electrode is diminished.

The electron-transfer mechanism

For the explanation of the electron-transport mechanism from SO molecules in the outer layers to the electrode two different

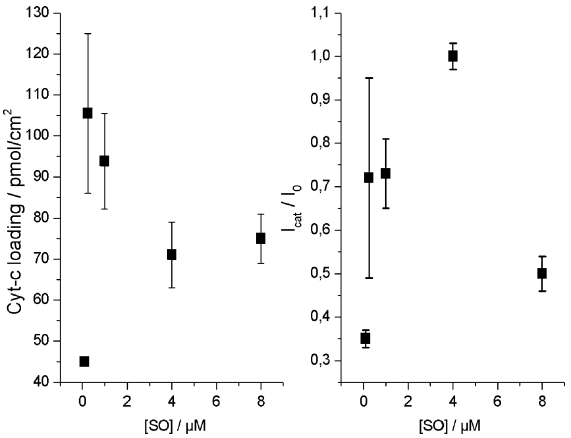

Fig. 5 Dependence of electroactive cyt c on the surface (left) and the ratio of catalytic oxidation and non-catalytic current (right) on the applied SO concentration in the protein mixture with 20 μM cyt c used for electrode preparation (Au/MU,MUA, (cyt c,SO/PASA)$_n$, $n = 4$).

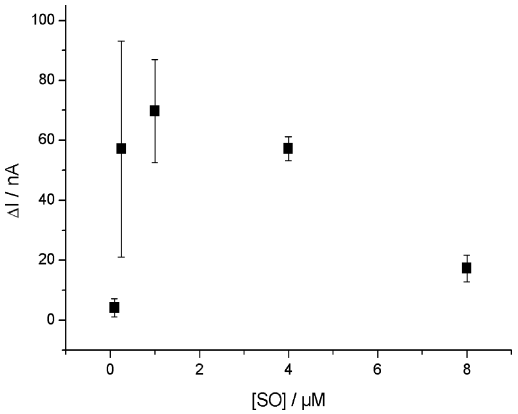

Fig. 6 Current generation dependent on the applied SO concentration in the protein mixtures containing 20 μM cyt c used for electrode preparation (Au/MU, MUA, (cyt c,SO/PASA)$_n$, $n = 4$, sulfite concentration = 1 mM).

models can be taken into account. Either the cyt c–cyt c ET may deliver the electrons to the electrode with the PASA acting as a simple "glue" in between the different layers, or the polyelectrolyte could act as a "wire" suitable for the ET, since under defined conditions it has a conducting function.[26,27] However, it does not show any redox activity in the potential range used here, *i.e.* no redox peaks were found in cyclic voltammograms for PASA in solution without cyt c and SO (see ESI†, Fig. S1). For cyt c multilayers an ET *via* cyt c was assumed, supported by experiments with apo–cyt c.[19,20] In the cyt c-SO multilayer system described here an additional enzyme is involved. Following the catalytic behaviour and comparing the rate constants for the individual processes (sulfite–SO, SO–cyt c, cyt c–cyt c, cyt c–electrode) may help to understand the mechanism. Looking at the system under catalysis, as in Fig. 4, a clear dependence of the oxidation current on the sulfite concentration in solution was detected. Additionally, as shown in Fig. 3, by increasing the number of bilayers the SO response, *i.e.* I_{cat}, is linearly growing. This supports that the velocity with which the electrons are

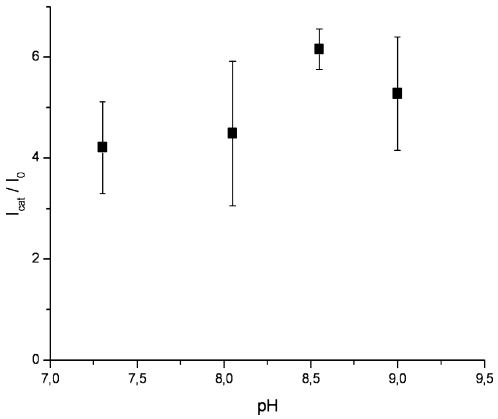

Fig. 7 Dependence of the ratio of oxidative catalytic and non-catalytic current on the pH of the assay buffer for a 4 bilayer electrode.

transferred from the sulfite to the electrode remain almost unchanged with the increasing distance.

These findings may be explained by a limitation of the whole process by SO activity rather than by cyt c–cyt c self-exchange or interfacial ET. This hypothesis is further supported by the fact that the multilayer is also showing the same pH optimum (Fig. 7) as the enzyme in solution.[28] Furthermore, the catalytic current was not reduced when three additional cyt c layers where pre-assembled on gold below the SO–cyt c layers.

The reaction control in the multi-protein assembly may be on the catalytic Moco site, the intramolecular Mo to Fe ET or due to SO–cyt c ET restriction. The situation in solution is different. When comparing the homogeneous ET rate constants in solution (Table 1) the slowest process is the cyt c self-reduction.

The SO activity was measured spectroscopically in solution following the reduction of cyt c at 550 nm. The ET rate from SO to cyt c (heme turnover rate) was calculated from electrochemical data obtained in solution by the method of Nicholson and Shain for an ECE mechanism.[29] A MU/MUA modified gold electrode was used to record the CVs with 50 μM of cyt c and different concentrations of SO at several scan rates (v). The ratio between catalytic (I_{cat}) and uncatalysed (I_0) currents is proportional to the square root of k_{cat}. In the following equation n is the number of electrons exchanged, R the universal gas constant, F the Faraday constant and T the temperature.

$$(I_{cat}/I_0)^2 = \lambda = k_{cat}(RT/nF)/v \quad (2)$$

$$k_{cat} = k[SO] \quad (3)$$

The value of the second-order reaction rate constant calculated by this approach at pH 8.5 is $k = 4.47 \pm 0.13$ μM^{-1} s^{-1}, which is close to the literature value of 2.53 μM^{-1} s^{-1} found at pH 7.4.[11] The catalytic efficiency and the SO–cyt c electron-transfer rate are three orders of magnitude larger than the cyt c–cyt c self exchange rate in solution.[30] In the protein–PASA-network the transfer of electron to the electrode is not rate limiting. Also, the local protein concentrations are very high and the heterogeneous ET rate constant of cyt c is about 75 s^{-1} and 2 s^{-1} for one[20] and eight layers, respectively. The discrepancy between homogeneous and heterogeneous case may be due to

a dramatic decrease of SO-turnover rate by matrix effects or an enhanced cyt c–cyt c self-exchange rate.

For the cyt c reduction rate enhancement two different factors may be taken in account: a reduction by superoxide eventually formed by SO and the role of PASA.

Other molybdenum containing enzymes, such as xanthine oxidase, were shown to react with molecular oxygen as an electron acceptor. The oxidation at the active site is then accompanied by release of superoxide anion radicals.[31]

These rather small molecules, which can easily diffuse through the multilayer, react quickly with heme iron. In this case the mechanism of ET would involve a chemical reaction[32] rather than a direct ET, although in the net reaction it would not make a difference. In order to verify that the catalytic current observed in the present study is based on direct protein–protein interaction, the response of a SO-cyt c multilayer was tested in the presence of the same substrate concentration in a nitrogen-purged and air-saturated buffer, however, no difference has been found. Furthermore, sulfite oxidation was recorded in an amperometric experiment (at +100 mV) with SO, cyt c and in the presence of superoxide dismutase (SOD). SOD (EC 1.15.1.1.) is one of the most effective superoxide scavengers. After sulfite was introduced into the electrochemical cell, a large increase in the oxidation current was obtained (Fig. 8). To remove the superoxide eventually produced, a SOD (1kU) was twice sequentially added. No effect on the oxidation current was detected. From this experiment it is evident that superoxide radicals are not involved in the signal transfer here.

PASA facilitates most likely, an enhancement of the cyt c–cyt c self-reduction rate. This may be due to an increased probability for productive collisions in the densely packed assembly because the conductive negatively charged polyelectrolyte in parallel increases the dielectric constant of the medium surrounding cyt c and reduces the heme edge-to-edge distance by interacting with the positively charged His-18 residues, which are important for establishing the optimum distance.[34] This is also in correspondence with earlier reports on long distance ET in cyt c–PASA multilayers.[19,20] Thus the functional assembly can be schematically illustrated (Fig. 9).

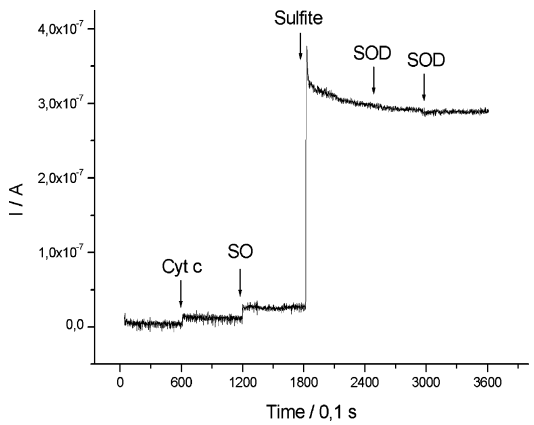

Fig. 8 Amperometric *I–t* curve of a MU,MUA modified gold electrode at +100 mV in potassium phosphate 5 mM pH 7, as solution, and successive additions of 20 μM cyt c, 2 μM SO, 1 mM sulfite and twice with 1kU SOD.

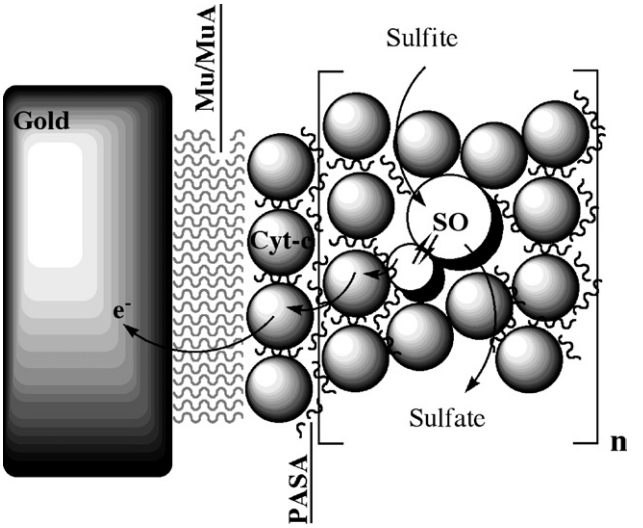

Fig. 9 Schematic illustration of structure and electron transport in the cyt c–SO multilayer. After the oxidation of the sulfite to sulfate occurring at the Moco domain of the SO (white balls), the electrons are first delivered to the heme b5 domain and then transferred to the closest molecule of cyt c, which transfers electrons further to the electrode *via* other cyt c molecules while re-oxidizing itself.

Summary and conclusions

For the first time a SO–cyt c multilayer was successfully formed as a fully electroactive assembly with the help of a self-assembly process and the polyelectrolyte PASA. The proteins, which are communicating *via* direct ET, constitute an artificial ET chain and have been immobilized from a protein mixture. The accumulation of the mass on the surface of a gold electrode has been followed by QCM and cyclic voltammetry showing for each deposition round an increase in mass and electroactive cyt c. The multilayer shows catalytic activity for the oxidation of sulfite – equally in the presence and absence of oxygen. The building process of the assembly was optimized by changing the solution pH and the ratio of the SO–cyt c mixed solution, in order to get the largest oxidation current in the presence of sulfite. Increasing numbers of biprotein layers result in a linear increase of both: electroactive cyt c amount and catalytic sulfite oxidation.

The SO–cyt c multilayer has been characterized calculating the apparent K_m^{app} for sulfite. The value was found to be significantly increased compared to the situation with the enzyme in solution, which gives information on the changed diffusion conditions of substrate within the assembly.

From earlier experiments several theories have been proposed to explain the electron transfer in such artificial protein assemblies. The two-protein multilayer approach described in the present work constitutes a big advantage not only for applications, but also, as an instrument for characterizing the ET mechanism. A limiting SO–cyt c ET and an active role of PASA are derived from the catalytic behaviour of the multilayer electrode and the reaction rate constants determined for the components in solution. Additionally the absence of a superoxide-mediated electrons flux has been proven.

Finally a wide range of applications can be imagined for the multilayer technology, *e.g.* SO–cyt c multilayer electrode could act as an anode in a bio-fuel cell and furthermore such

multilayers could be exploited as a biosensor for the detection of sulfite, which is used as a preservative in wine and other foodstuffs.

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References

1 D. Voet, J. G. Voet, *Biochemistry*, Wiley, New York, 3rd edn, 2004.
2 S. D. Minteer, B. Y. Liaw and M. J. Cooney, *Curr. Opin. Biotechnol.*, 2007, **18**, 228–234.
3 S. Rodriguez-Mozaz, M. J. Lopez de Alda and D. Barcelo, *Anal. Bioanal. Chem.*, 2006, **386**, 1025–1041.
4 G. Justin, Y. Zhang, M. Sun and R. Sclabassi, *Conf. Proc. IEEE Eng. Med. Biol. Soc.*, 2004, **6**, 4096–4099.
5 I. Willner, B. Willner and E. Katz, *J. Biotechnol.*, 2002, **82**, 325–355.
6 F. A. Armstrong, *Curr. Opin. Chem. Biol.*, 2005, **9**, 110–117.
7 J. L. Johnson and K. V. Rajagopalan, *J. Biol. Chem.*, 1977, **252**, 2017–2025.
8 C. A. Temple, T. N. Graf and K. V. Rajagopalan, *Arch. Biochem. Biophys.*, 2000, **383**, 281–7.
9 H. R. Brody MS, *Biochemistry*, 1999, **38**, 6668–6677.
10 E. P. Sullivan Jr., J. T. Hazzard, G. Tollin and J. H. Enemark, *Biochemistry*, 1993, **32**, 12465–12470.
11 E. E. Ferapontova, T. Ruzgas and L. Gorton, *Anal. Chem.*, 2003, **75**, 4841–4850.
12 J. P. Hart, A. K. Abass and D. Cowell, *Biosens. Bioelectron.*, 2002, **17**, 389–394.
13 L. A. Coury Jr., B. N. Oliver, J. O. Egekeze, C. S. Sosnoff, J. C. Brumfield, R. P. Buck and R. W. Murray, *Anal. Chem.*, 1990, **62**, 452–458.
14 L. Yang, R. W. Murray and L. A. Coury, *Anal. Chem.*, 1993, **65**, 242–246.
15 R. W. Murray, L. A. Coury, J. L. Johnson and K. V. Rajagopalan, *J. Phys. Chem.*, 1991, **95**, 6034–6040.
16 L. Yang, L. A. Coury and R. W. Murray, *J. Phys. Chem.*, 1993, **97**, 1694–1700.
17 F. W. Scheller, U. Wollenberger, C. Lei, W. Jin, B. Ge, C. Lehmann, F. Lisdat and V. Fridman, *J. Biotechnol.*, 2002, **82**, 411–424.
18 W. Jin, U. Wollenberger, E. Kärgel, W. H. Schunck and F. W. Scheller, *J. Electroanal. Chem.*, 1997, **135**, 433–440.
19 M. K. Beissenhirtz, F. W. Scheller, W. F. Stocklein, D. G. Kurth, H. Möhwald and F. Lisdat, *Angew. Chem., Int. Ed.*, 2004, **43**, 4357–4360.
20 M. K. Beissenhirtz, F. W. Scheller and F. Lisdat, *Anal. Chem.*, 2004, **76**, 4665–4671.
21 N. Loew, F. W. Scheller and U. Wollenberger, *Electroanalysis*, 2004, **16**, 1149–1154.
22 R. Dronov, D. Kurth, H. Möhwald, F. Scheller and F. Lisdat, *Angew. Chem., Int. Ed.*, 2008, DOI: 10.1002/anie.200704049.
23 C. Feng, R. V. Kedia, J. T. Hazzard, J. K. Hurley, G. Tollin and J. H. Enemark, *Biochemistry*, 2002, **41**, 5816–5821.
24 B. Ge and F. Lisdat, *Anal. Chim. Acta*, 2002, **454**, 53–64.
25 S. K. Kitani, K. Iwai and S.Ito, *Synth. Met.*, 1999, **102**, 1171–1172.
26 Z. H. W. Jiang Yue, K. R. Cromack, A. J. Epstein and A. G. MacDiarmid, *J. Am. Chem. Soc.*, 1991, **113**, 2665–2671.
27 X. L. Wie, Y. Z. Wang, S. M. Long, C. Bobeczko and A. J. Epstein, *J. Am. Chem. Soc.*, 1996, **118**, 2545–2555.
28 R. M. MacLeod, W. Farkas, I. Fridovich and P. Handler, *J. Biol. Chem.*, 1961, **236**, 1841–1846.
29 R. S. Nicholson and I. Shain, *Anal. Chem.*, 1965, **37**, 178–190.
30 R. K. Gupta, *Biochim. Biophys. Acta*, 1973, **292**, 291–295.
31 P. Orth, C. Galbusera, D. Fedida and T. Spector, *Biochem. Pharmacol.*, 2006, **71**, 1747–1752.
32 R. Dronov, D. Kurth, H. Möhwald, F. Scheller and F. Lisdat, *Electrochim. Acta*, 2007, **53**, 1107–1113.
33 R. M. Garrett and K. V. Rajagopalan, *J. Biol. Chem.*, 1996, **271**, 7387–7391.
34 R. A. Marcus and N. Sutin, *Biochim. Biophys. Acta*, 1985, **811**, 265–322.
35 E. Katz, D. D. Schlereth and H. L. Schmidt, *J. Electroanal. Chem.*, 1994, **367**, 59–70.
36 E. Katz, A. F. Bückmann and I. Willner, *J. Am. Chem. Soc.*, 2001, **123**, 10752–10753.
37 I. Willner and B. Willner, *Trends Biotechnol.*, 2001, **19**, 222–230.

