Multilayer architectures of enzymes on electrodes for sensorial application – influence of the building blocks on the assembly behaviour

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ABSTRACT

Proteins can be advantageously combined with electrodes in such a way that direct electron transfer becomes possible. This can be applied for sensorial use of these protein electrodes. One approach to increase the sensitivity can be seen in the arrangement of multiple protein layers on the sensing surface. In this case, however, electrochemical communication has to be ensured. It can be shown that the small redox protein cytochrome c can be assembled in layers on modified gold electrodes resulting in fully electro-active systems. Different second building blocks can be used for the layer-by-layer assembly process. Besides natural and artificial polymers this can also be nanoparticles. These layered systems can be combined with enzymes leading to protein electrodes sensitive for the respective substrate. For the communication between the immobilised protein molecules not only shuttle molecules can be applied, but conditions can be found that direct electron exchange becomes feasible.

Keywords: cytochrome c, bi-protein electrodes, direct protein-protein electron transfer, biosensor, layer-by-layer-assembly strategy

1 INTRODUCTION

Different principles have been developed for the combination of proteins with electrodes. Particularly advantageous are systems in which the communication is achieved without the use of products of the biocatalytic reaction or mediating shuttle molecules. Such bioelectrodes of the so-called third generation are promising analytical tools since they allow well defined analyte detection [1,2]. Here conversions at the biocatalysts are directly transfered into an electrical signal. Different approaches exist for enhancing the sensitivity of such biosensors. One way focuses on the amount of active protein molecules, thus one has to go beyond the monolayer arrangement of redox proteins on electrode surfaces.

Cytochrome c is a small redox protein which has been extensively studied with respect to direct electrode communication [3-5]. It has also been shown to be beneficial for the sensorial detection of superoxide – one of the reactive oxygen species – particularly relevant in the medical area [6,7]. Several advantages can be seen in the sensorial application of this protein on electrodes: well-defined redox chemistry, natural function as a redox shuttle in the respiratory chain, rather small molecule, dipol character with a positive patch at the heme edge, stability on surfaces and in solution. With this molecule it could be shown that a successful layer-by-layer arrangement on electrodes is feasible. Furthermore, it can be shown that by use of a weak polyelectrolyte such as sulfonated polyaniline full electroactivity can be ensured in these multiple layer systems. This means that all deposited molecules are able to take part in the electron transfer process and thus can be oxidized or reduced [8]. Several studies have been performed dealing with the mechanism behind this electron transport through the layered protein assembly. AFM measurements show that rather flat layers are deposited and the surface can be completely recharged after incubation in the polyelectrolyte or protein solution respectively [9]. Comparative QCM and CV measurements indicate that the deposited mass correlates with the electro-active protein amount [10]. SERS studies reveal a rather undisturbed heme environment of cyt c in the multilayer network and that cyt c is rather fastly oxidised/reduced [11]. All together this indicates that efficient interprotein electron transfer takes place and that it is the dominating process of electron transfer within the layered system although sulfonated polyaniline itself possesses some redox activity [12].

2 EXPERIMENTAL

2.1 Materials

11-Mercaptoundecanoic acid (MUA), 11-mercapto-1-undecanol (MU), horse heart cytochrome *c* (cyt *c*), bilirubin oxidase (BOD), DNA from calf thymus, and lactose are purchased from Sigma-Aldrich (Steinheim, Germany), di-potassium hydrogen phosphate, potassium di-hydrogen phosphate are provided by MERCK (Darmstadt, Germany), and gold-wire electrodes by Goodfellow (Bad Nauheim, Germany), gold chips for QCM are delivered by QSense (Frölunda, Sweden). All solutions were prepared in 18 MΩ Millipore water (Eschborn, Germany). CDH from *Trametes villosa* was a gift from R. Ludwig (BOKU Vienna) and human sulfite oxidase (SOx) from S. Leimkühler (Uni Potsdam). Silica nanoparticles were prepared according to [16].

2.1 Electrode preparation

Gold-wire electrodes were cleaned by incubation in Piranha solution (3:1 H_2SO_4/H_2O_2) for 10min. The electrodes were washed with Millipore water. For the construction of multilayers the electrodes were first modified by incubation for 48h in 5mM 3:1 solution of MU/MUA. CDH, cyt *c* and SiNPs or DNA are immobilized by adsorption. The cyt *c* monolayers were prepared by incubation of the electrodes in 30µM cyt *c* in potassium phosphate buffer (KPP) 5mM pH7 for 2h. For the assembly with SiNPs a premixed protein solution was made in potassium phosphate buffer (5mM, pH 7), which contained cyt *c* (20µM) and CDH (2µM) in a 10:1ratio and for the assembly with DNA a premixed protein solution was made in potassium phosphate buffer (0.5mM, pH 5), which contained cyt *c* (20 µM) and CDH (2µM) in a 10:1 ratio. The assembly of CDH•cyt *c*/SiNPs layers has been performed by alternating incubations of the cyt *c* monolayer electrode in CDH•cyt *c* (1:10) and SiNPs (5.0 mg•mL-1) solutions for 10min per step. Each of the 10-min long adsorption steps of SiNPs (5.0mg•mL-1) and a premixed CDH•cyt *c* (1:10) solution was followed by rinsing the electrodes with 5mM KPP pH7, and the assembly of CDH•cyt *c*/DNA layers has been performed by alternating incubations of the cyt *c* monolayer electrode in CDH•cyt *c* (1:10) and DNA (0.2mg•mL^{-1}) solutions for 10min per step. Each of the adsorption steps of DNA and CDH•cyt *c* was followed by rinsing the electrodes with 0.5mM KPP pH5.

2.2 Electrochemical measurements

All electrochemical measurements were carried out in a custom made 1mL cell using an Ag/AgCl/1M KCl reference (Biometra, Germany) and Pt-wire counter electrode. The working electrodes were modified gold wires (∅ 0.5mm) obtained from Goodfellow (Bad Nauheim, Germany), which are modified according to the procedures described. Cyclic voltammetry (CV) was carried out with CH Instruments CHI 660D device (Austin Texas, USA). Scan rates were varied between 0.005 and 50V•s^{-1}, but a scan rate of 5mV•s^{-1} was used to record catalytic currents, 100 mV•s^{-1} was applied for determination of the cyt *c* concentration. All measurements were performed at room temperature, 25 °C.

2.3 QCM measurements

A Q-Sense-D E4 piezoelectric instrument (QSense, Västra Frölunda, Sweden) was used for the quartz crystal microbalance measurements. A clean gold covered quartz sensor chip (5 MHz, QSense, Västra Frölunda, Sweden) was incubated in ethanolic solution containing 5 mM MUA/MU (1:3) for 24h, then rinsed with water and mounted into the QCM flow system. The solutions containing SiNPs or DNA, cyt *c* and CDH•cyt *c* of above given concentrations were successively pumped through the cell for 10min with 5min of buffer flow in between, at a flow rate of 25µL/min. We estimated the mass increase [Δm (ng)] from the QCM frequency shift [Δ*f* (Hz)] of the fixed films by using the Sauerbrey equation. According to this a layer mass increase can be estimated. Since measurements have been performed in solution, these values are estimates due to unknown amount of bound water.

3 RESULTS AND DISCUSSION

3.1 Cyt c multilayers with different building blocks

In order to form multilayer architectures different second building blocks can be introduced during the assembly process. The layer built up starts with cytochrome c adsorbed on a negatively charged thiol layer (mercaptoundecanoic acid/mercaptoundecanol) on gold allowing for a quasi-reversible conversion of the redox protein in a monolayer [13]. The surface has to be recharged by the second building block of the assembly in order to allow for subsequent protein adsorption. Besides the polyelectrolyte sulfonated polyanilline (PASA) modified gold nanoparticles can be applied. The negative charge has been introduced by mercaptopropionic acid. This leads to a rather continous increase in mass and electroactivity with every deposition round [14]. Alternatively DNA as a natural polyelectrolyte has been applied. At pH 5 stable multilayer architectures are formed. However, these systems are rather unique: they show an enormous mass increase with increasing layer number and a very high surface loading. For example a 6 layer electrode exhibits an electro-active cyt c amount of ~320 pmol/cm^2. This high loading is accompanied by a rather rough surface [15]. The reason for this behaviour has to be attributed to the rather rigid properties of a ds DNA helix with a rather high persistence length.

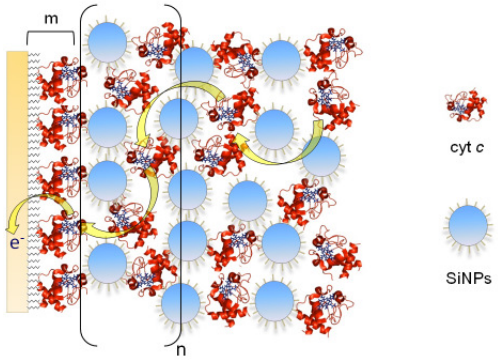

Figure 1: Scheme of a cytochrome c multilayer electrode using carboxy-mofified silica nanoparticle as building block during the layer by layer formation of the protein assembly

In all these cases the second building block has some properties that it may be involved in the electron transfer between the cyt c molecules. Therefore, another strategy has been developed using a non-conducting building block: silica nanoparticle (SiNP) - the surface of which is modified with carboxy groups [16]. Also in this case multilayer formation has been shown to be feasible. This has been verified by QCM measurements. A scheme of the multilayer assembly is given in Figure 1.

Fully electro-active cyt c multilayers can be obtained. The formal potential for cyt c is around 0mV vs Ag/AgCl. Figure 2 illustrates the cyclic voltamograms of electrodes with different numbers of cyt c layers. Interestingly a rather small peak sepration has been found here indicating rather fast electron transport through the system. The use of a non-conducting material demonstrates for the first time that mediating properties of the second building block of the multilayer assembly is not a pre-requisite for the formation of active systems. The dominating role of protein-protein electron transfer is clearly verified.

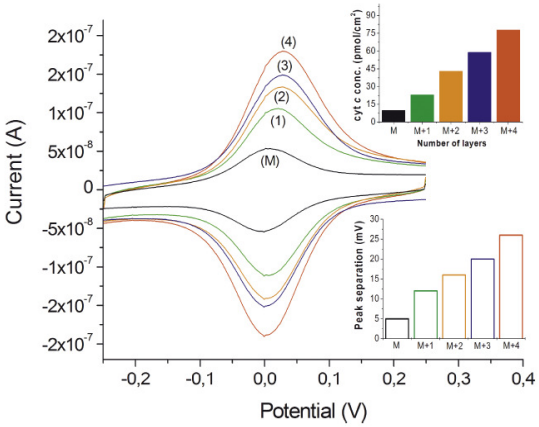

Figure 2: Cyclic voltammetry of a Au/MUA,MU/cytc monolayer electrode (M) and multilayer electrodes prepared from carboxy-modified silica nanoparticles and cyt c (1-4 bilayers on top of the monolayer). The insets show the dependence of the electro-active surface amount and the peak separation at a scan rate v=100mV/s on the number of cyt c layers on the electrode. (20mM KPP pH7)

3.2 Coupling of enzymes with cyt c multilayers resulting in sensorial signal chains

The approach of arranging proteins in more complex structures follows natural examples when ptroteins are assembled in larger complexes ensuring defined and interference-free signal transfer. Cyt c is a redox protein and has no specific catalytic ability (except some pseudoperoxidative activity). Thus, it would be beneficial when enzymes could be coupled with cyt c assemblies. Most straigthforward would be an approach in which the assembled proteins can interact in such a way that reactions occuring at the catalytic site can be transferred to neighboring protein molecules which may act as electron shuttles. This concept has been first exemplified with the embeddment of billirubin oxidase (BOD) into cyt c multilayers. Although both proteins are not natural reaction partners the electron transfer from reduced cyt c towards BOD can be confined to the immobilised state and ensured within multiple layers on electrodes [17]. The concept is shown in Figure 3. This electrode is sensitive to molecular oxygen as the final electron acceptor at the BOD and the reduction current as the sensor signal is dependent on the number of deposited BOD/cytc layers on the electrode. This allows a tuning of the sensing properties during the preparation of the bi-protein electrode.

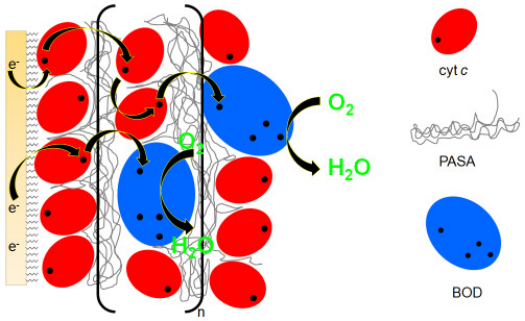

Figure 3: Schematic illustration of the working principle of a BOD, cyt c multilayer system on electrodes.

Not only BOD can be combined succesfully with cyt c multilayers ensuring electrochemical connection of the biocatalyst to the electrode by means of the cyt c network. Sulfite oxidase (SOx) and cellobiose dehydrogenase (CDH) are further examples. For sulfite oxidase it can be shown that incorporation of the enzyme in a cyt c environment is possible by means of a polyelectrolyte [18], but since these proteins are here natural reaction partners, it can be demonstrated that interaction can be strong enough to allow layer formation without the help of a second building block [19]. At pH5 the charge situation on the surface of SOx and cyt c is opposite allowing for the development of an assembly strategy. Also here sensitivity can be tuned by the numbers of layers. In contrast to BOD the direction of electron transfer is reversed: Cyt c acts here as electron acceptor from the reduced enzyme after substrate conversion. This may also illustrate the generality of the approach.

CDH is an enzyme with two subunits and able to accept cyt c as reaction partner [20]. It can be integrated into cyt c multilayers. This has been tested with systems based on silica nanoparticles and DNA. It turns out that glycolisation may be an obstacle when proteins shall be efficiently immobilised by using electrostatic forces and that electron transfer can be hindered too. By applying deglycosylated enzymes this can be overcome and functional systems can be obtained both with DNA and silica nanoparticles as building block [21]. However, the catalytic response is found to be higher in case of silica based multilayer systems. This appears although the cyt c loading is higher for DNA-based layer systems. Fig. 4 illustrates the catalytic

current behaviour of a CDH, cyt c/ silica multilayer system in dependence on the lactose concentration in solution showing the defined concentration dependence of the electrode signal.

Since even CDH molecules, which are immobilised in layers far from the electrode surface, are electrochemically connected via cyt c, the sensitivity can be tuned by the number of bi-protein layers. This will only reach limitations when the diffusion of the substrate becomes limiting, but has not been observed untill 5 layers.

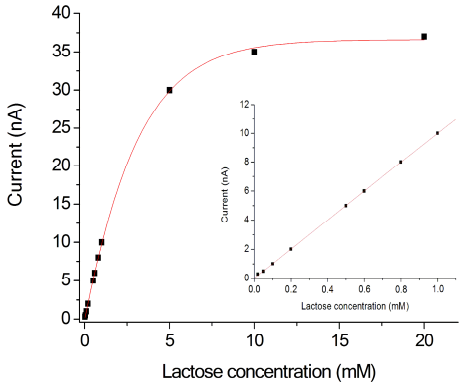

Figure 4: Dependence of the catalytic current of a Au-MUA/MU-cyt *c*-[SiNP-cyt *c*•dCDH]$_4$ multilayer electrode on the lactose concentration. (20mM KPP pH 4,5)

4 CONCLUSIONS

Proteins can be coupled with electrodes not only in a monolayer fashion but also arranged stably in multiple layers. It can be shown that communication between the immobilised protein molecules and the electrode can be achieved by protein-protein electron transfer avoiding bound or free-diffusing shuttle molecules. This has been exemplified with the redox protein cyt c. Particularly advantageous for the layer formation are non-conducting silica nanoparticles. But also DNA as natural poly-electrolyte is feasible resulting in very high protein loadings. However, in combination with enzymes these systems are less efficient than nanoparticle based multilayers with respect to the catalytic current output. The systems introduced reflect natural examples of signal cascades and are expected to have importance in bioanalysis but also for the better understanding of natural signal pathways.

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