Communication

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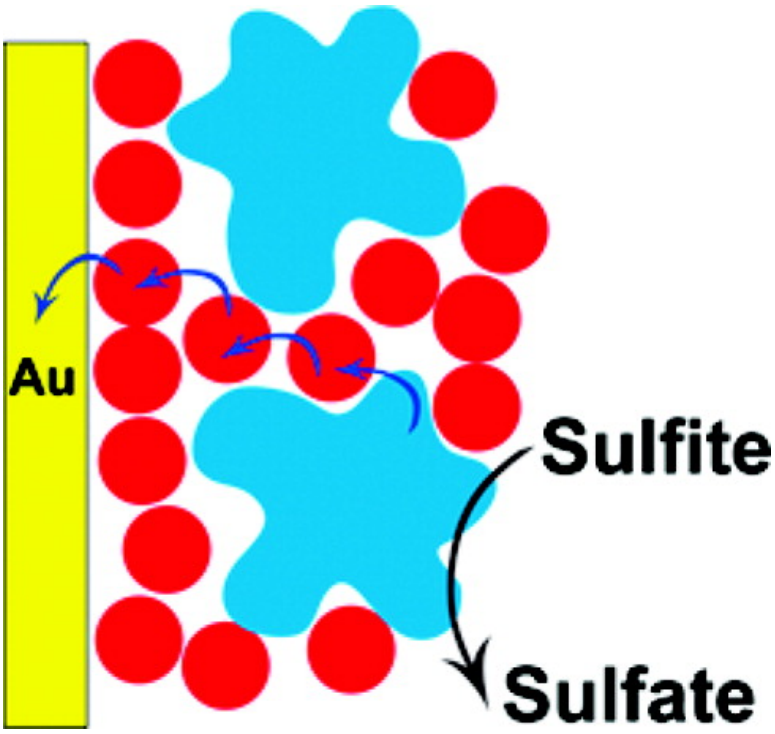

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Layer-by-Layer Arrangement by Protein−Protein Interaction of Sulfite Oxidase and Cytochrome *c* Catalyzing Oxidation of Sulfite

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Functional protein multilayer assemblies are receiving broad scientific attention as simple and flexible model systems in bioelectronics and for biomimetic signal transfer.[1] Polyelectrolyte multilayer (PM) arrangements with embedded proteins attract particular interest owing to simplicity of fabrication, tunable architecture, and general versatility with respect to the functional components.[2] Although a number of PM designs have been reported,[3] all of these structures are stabilized by a polyelectrolyte matrix. Usually the scaffolding matrix is composed of an electrochemically inert polymer which stabilizes the arrangement.[2,4] Recently reported assemblies combining cytochrome *c* with enzymes and using sulfonated polyaniline (PASA) as a counter-polyelectrolyte show high efficiency in electron transfer throughout the multilayer network. Electron exchange between the cyt *c* molecules was proposed as a dominating mechanism, but the role of the polymer in the electron transfer process has not been solved completely. Therefore, avoiding the use of a scaffolding polyelectrolyte is one way to shed more light on the electron transfer mechanism within multiprotein arrangements.

Here we describe a novel strategy for multiprotein layer-by-layer self-assembly combining the redox protein cytochrome *c* (cyt *c*) with the enzyme sulfite oxidase (SOx) without use of any additional polymer. Electrostatic interactions between these two proteins with rather separated isoelectric points during the assembly process from a low ionic strength buffer were found to be sufficient for layer-by-layer deposition of both components. Mediator-free electron transfer of the enzyme within the film is achieved by co-immobilization of the enzyme and the redox protein from a mixture, rather than pure solutions.

We assembled the horse heart cyt *c* and human SOx (EC 1.8.3.1) by sequential incubation steps of a cyt *c* monolayer electrode in solutions of SOx and cyt *c* prepared in 0.5 mM potassium phosphate buffer pH 5.0. The monolayer electrode constructed by electrostatic adsorption of cyt *c* onto an OH- and COOH-terminated mixed alkanethiol promoter layer shows efficient protein−electrode communication.[5]

As a starting experiment, we assembled SOx/cyt *c* multilayers from solutions of pure SOx (10 μM) and cyt *c* (20 μM) to study the deposition of these two proteins. Quartz crystal microbalance (QCM) confirmed mass accumulation at the surface with each deposition cycle (see Supporting Information Figure S1). Although SOx exceeds the mass of cyt *c* by about a factor of 5, the enzyme still can bind to the cyt *c* modified surface and facilitate further adsorption of cyt *c*.

However, the voltammetric signal of cyt *c* within the assemblies formed by sequential deposition shows practically no dependence on the number of layers. This behavior indicates that electron transfer between cyt *c* in the monolayer and cyt *c* separated by a SOx layer is hindered. Assuming that the large enzyme molecules disrupt communication between different cyt *c* layers, we co-adsorbed SOx together with cyt *c* from a mixture in order to improve electrochemical characteristics of the film.

In accordance with this method, SOx/cyt *c* arrangements were built by alternating incubation of a cyt *c* monolayer electrode into a SOx/cyt *c* mixture and a pure cyt *c* solution. QCM experiments showed an increase in the deposited protein mass after each deposition cycle, and AFM investigation proves the thickness increase (see Supporting Information Figures S2 and S4).

The SOx/cyt *c* arrangement prepared from the mixture containing 10 μM SOx and 1 μM cyt *c* adsorbed against pure 20 μM cyt *c* showed a pronounced electrocatalytic effect for oxidation of sodium sulfite. Figure 1 demonstrates the electrode response upon addition of sulfite to the solution in the micromolar concentration range. Sulfite oxidation takes place at the SOx molecules, thus in the presence of the substrate, most of the enzyme is reduced. A catalytic current is generated due to subsequent oxidation of SOx by cyt *c* molecules, followed by electron transfer toward the electrode. Long-range electron transfer process has been previously reported for a PM arrangement of cyt *c* and PASA only.[6] Here two possible mechanisms were considered: (i) electron transfer occurs by direct interaction between neighboring cyt *c* molecules, while the polyelectrolyte is responsible for stabilization of the arrangement; or (ii) cyt *c* can be wired by PASA, which under specific conditions can be a conductive polymer. However, in the current case, long-range electron transfer is observed in a polymer-free system, which may give an additional argument for a face-to-face electron hopping between cyt *c* molecules as a dominating electron transfer mechanism.

Cyclic voltammograms of the assemblies containing a different number of layers (Figure 2), recorded in the presence of 1 mM sodium sulfite, show a linear increase of the catalytic current. This experiment not only confirms the QCM and AFM data for the assembly growth with each deposition cycle but also indicates that the two proteins that are natural reaction partners are able to maintain efficient electronic communication being co-immobilized in multiple layers on the electrode. Since the system combines an electron transfer protein with an enzyme, we assume that cyt *c* within the arrangement is responsible for electron shuttling between

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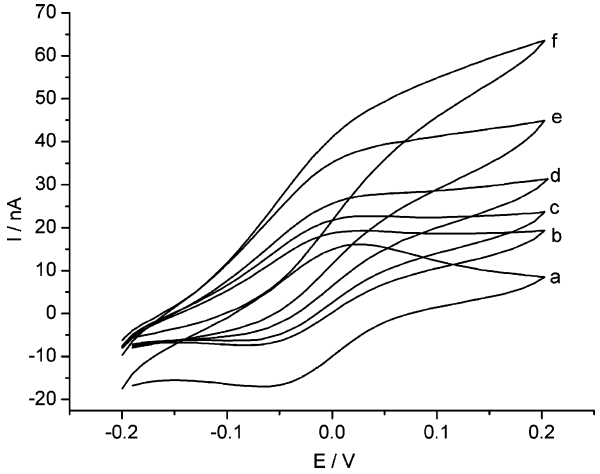

Figure 1. Cyclic voltammograms of Au-MUA/MU-{(SOx/cyt *c*-cyt *c*)}$_8$-SOx/cyt *c* electrode measured in (a) the absence of sulfite, (b) 60 μM, (c) 125 μM, (d) 250 μM, (e) 0.5 mM, and (f) 1 mM Na_2SO_3; scan rate 100 mV·s^{-1}.

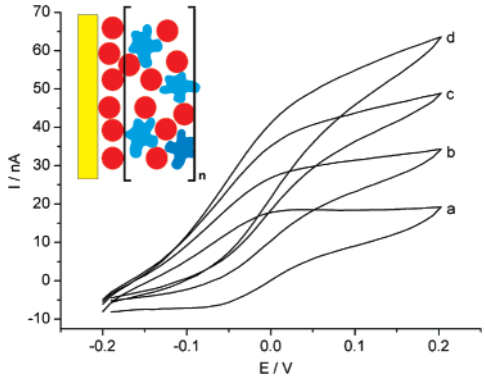

Figure 2. Catalytic response of Au-MUA/MU-{(SOx/cyt *c*)-cyt *c*}$_n$-SOx/cyt *c* electrode in 1 mM Na_2SO_3: (a) $n = 2$, (b) $n = 4$, (c) $n = 6$, (d) $n = 8$; scan rate 100 mV·s^{-1}, pH 7.0. (Inset) Electrode scheme: red circles = cyt *c*, blue objects = SOx.

the electrode and the enzyme where the catalytic reaction takes place. In this case, variation of the relative amount in the SOx/cyt *c* mixture should have a profound effect on the electrochemical response of the arrangement. Indeed, assemblies built up using a SOx/cyt *c* mixture with lower enzyme concentration showed smaller catalytic response (Figure 3 inset). At the same time, electrodes prepared with a high relative amount of enzyme ([SOx]/[cyt *c*] > 25) showed a low catalytic efficiency. Therefore, electrocatalytic performance of the arrangement requires (i) a sufficient SOx surface concentration in order to generate a catalytic current, and (ii) that the amount of cyt *c* within the assembly is high enough to provide a long-range electron transfer to connect the enzyme to the electrode. Notably, the fast reaction of cyt *c* with SOx in solution ($k = 4.47 \pm 0.13$ μM^{-1} s^{-1}, pH 8.5) is preserved in the immobilized state since the catalytic effect can be observed even at scan rates up to 500 mV·s^{-1} (Figure 3).

The catalytic current of the multilayer electrode follows the activity of SOx at different pH with a maximum response at pH 7, which is slightly shifted compared to the activity in solution (pH 8.5, see also Supporting information Figure S3). The current depends on the sulfite concentration in the range from 20 μM to 2 mM with an apparent Michaelis−Menten constant K_M of about 310 μM. This experiment also indicates that the electron transfer from the enzyme to the electrode is fast enough to follow the catalytic reaction and, thus, is not the rate-limiting step.

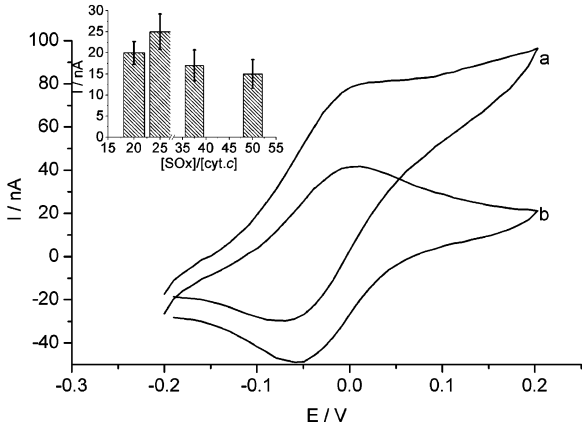

Figure 3. Cyclic voltammograms showing catalytic response of Au-MUA/MU-{(SOx/cyt *c*)-cyt *c*}$_8$-SOx/cyt *c* electrode scanned at 500 mV·s^{-1} in 1 mM Na_2SO_3 (a) and the absence of sulfite (b). (Inset) Dependence of the catalytic current of Au-MUA/MU-{(SOx/cyt *c*)-cyt *c*}$_3$-SOx/cyt *c* electrode on SOx/cyt *c* ratio at a potential of +200 mV, error bars are derived from two measurements. Measurement performed at 5 mV·s^{-1} in the presence of 1 mM sulfite, pH 7.0.

In summary, we show that the layer-by-layer assembly of globular proteins is feasible without use of polymers as counter-polyelectrolyte. The assembly is made by co-adsorption of the enzyme SOx and the electron transfer protein cyt *c*. This arrangement shows a remarkable ability to transport electrons from the substrate in solution to the electrode over longer distances, taking advantage of direct interaction between the two functional biocomponents. Most importantly, the design does not require the use of additional redox mediators or a conventional polymer as a polyelectrolyte, which makes this approach interesting for the construction of third-generation biosensors.

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Supporting Information Available: Experimental procedure for assembly of SOx/cyt *c* films, Figure S1 (QCM of SOx adsorption against cyt *c*), Figure S2 (QCM of SOx/cyt *c* blend adsorption against cyt *c*), Figure S3 (pH dependence of the catalytic response), Figure S4 (AFM of the assembly stages). This material is available free of charge via the Internet at http://pubs.acs.org.

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